THE LUNG MICROBIOME IN CHRONIC AIRWAY DISEASE: DETERMINANTS AND CLINICAL IMPLICATIONS

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ABSTRACT

Chronic diseases of the lower airways are a leading cause of global morbidity and mortality. The current approach to the management of these patients relies on diagnostic labels based on criteria including clinical history, environmental exposures, and physiology. While these criteria help us to understand disease aetiology, they poorly describe the substantial interpersonal variation in disease trajectory and response to treatment. As most symptoms arise from the lower airways, an emerging approach to improve patient stratification is to precisely characterise the lower airway environment in a clinically informative manner. The pathophysiology of this environment is determined by complex disease traits, such as the degree and type of airway inflammation, mucus secretion, and microbial colonisation. However, the interactions between these traits, and how they reflect and contribute to lung pathophysiology are poorly understood.

With advances in sequencing technology, the improved ability to measure the lower airway microbiota can identify not only pathogenic organisms that contribute to disease, but also compositional characteristics of the microbiota that reflect the selective conditions of the airways. However, it is unknown whether microbiota analysis can provide insight into the complex lower airway environment and stratify patients in a clinically informative manner. It is also unknown what lower airway determinants select the microbiota and how this affects disease. This dissertation aims to explore these unknowns by measuring the effect of determinants of the lower airway environment on the microbiota composition and assessing how this correlates with clinical markers of disease.

Firstly, the selective effect of airway inflammation is explored in patients with persistent uncontrolled asthma. Neutrophilic inflammation, but not eosinophilic inflammation, was found to select a microbiota composition that has a low diversity and a high relative

abundance of taxa considered pathogenic. Secondly, the selective pressure of mucus composition is examined, where variation to mucus sugar expression is explored in relation to the lower airway microbiota in patients with bronchiectasis. Patients who display versatile sugar groups in mucosal secretions were found to select a microbiota dominated by pathogenic organisms, with important clinical consequences. Thirdly, the selective pressure of pharmaceutical treatment is assessed, through exploring the effect of long-term macrolide treatment on antibiotic resistance gene carriage and microbiota composition. To measure broad changes in resistance gene carriage, a novel shotgun metagenomic sequencing method was developed and tested. This identified that macrolides increase the carriage of both macrolide and tetracycline resistance genes. Through assessment of the selective effect of macrolides on microbiota composition, it was found that macrolides reduce microbiota diversity and the abundance of a key airway pathogen.

Together, the results of this dissertation demonstrate the potential clinical value of microbiota analysis to assess the characteristics of the lower airway environment. The selective pressures of airway inflammation type, mucosal sugar presentation, and macrolide treatment have profound effects on the airway environment and can contribute to disease through their ability to influence the composition of the airway microbiota. These findings represent important steps towards a precision medicine approach based on knowledge of an individual's disease characteristics.

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ABBREVIATIONS

ACQ6	Asthma control questionnaire 6 question format
AMAZES	Asthma and Macrolides: The Azithromycin Efficacy and Safety study
ANOVA	Analysis of variance
BLESS	The Bronchiectasis and Low-dose Erythromycin Study
CARD	Comprehensive antibiotic resistance gene database
CF	Cystic fibrosis
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CPM	Counts per million total reads
Ct	Threshold cycle
CT	Computer tomography
FEV_1	Forced expiratory volume in 1 second
FEV_1 %	Forced expiratory volume in 1 second, as a percentage of predicted value
FUT2	Gene encoding an α(1,2)-fucosyltransferase
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
HBA	Horse blood agar
HIV	Human Immunodeficiency Virus
ICS	Inhaled corticosteroids
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range

LABA	Long-acting beta agonist
LCQ	Leicester Cough Questionnaire
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis Effect Size
NTM	Non-tuberculous mycobacterial
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCO	Principal Coordinate
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PDPE	Physician defined pulmonary exacerbations
PEAR	Paired-End reAd mergeR
PERMANOVA	Permutational multivariate analysis of variance
QIIME	Quantitative Insights Into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RDP	Ribosomal data project
RSV	Respiratory syncytial virus
SA	South Australia
SABA	Short-acting beta agonist
SD	Standard deviation
sese	Homozygous non-secretor genotype
Sese	Heterozygous secretor genotype
SeSe	Homozygous secretor genotype
SGRQ	Saint George's respiratory questionnaire

SIMPER	Similarity of percentages
SNP	Single nucleotide polymorphism
SRA	Sequence Read Archive
STD	Standard deviation
TLR	Toll-like receptor
HRP-UEA1	Horseradish peroxidase conjugated *Ulex Europaeus* lectin 1

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DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

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CHAPTER 1: INTRODUCTION

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1.1 The Human Respiratory Tract

The human respiratory tract is comprised of the upper respiratory tract (including the nasopharynx and oropharynx) and the lower respiratory tract (including the trachea, bronchi, bronchioles, and alveoli), with the larynx forming the conduit between the two. The airways of the upper respiratory tract serve primarily to conduct humidified, filtered air to the lower airways, however they also form the interface with the gastrointestinal tract and the site where taste and smell are sensed. The lower airways branch out and narrow to maximise surface area at the terminal alveoli where gas exchange occurs.

Owing to the high exposure to the external environment, the respiratory tract surface is vulnerable to environmental insult, such as inhaled particles and pathogenic organisms. Insult to the respiratory tract is mitigated by the highly adapted mucous barrier that lines the respiratory tract as well as local immune responses. Collectively termed the respiratory mucosal immune system, this varies in function between the upper and lower respiratory tracts (Janeway et al., 2008).

In the upper respiratory tract, submucosal glands and goblet cells secrete mucin glycoproteins which hydrate to form a thick mucous lining approximately 10-15 μm thick

(Mercer et al., 1992; Wilson and Allansmith, 1976). This mucus forms a physical barrier separating the external environment from the underlying epithelia, trapping inhaled foreign particles. Mucus is continually turned over by ciliated epithelial cells that beat in a coordinated manner to move mucus towards the oropharynx where it is, most commonly, swallowed with saliva into the gastrointestinal system (Janeway et al., 2008).

In the lower respiratory tract, as the airways branch distally and become narrower, mucin production reduces, hence the coating mucus layer thins from 5 μm to 0.5 μM (Fahy and Dickey, 2010; Mercer et al., 1992). In healthy individuals, lower airway mucous clearance is effectively achieved through ciliary beating, however, owing to the greater distance to the oropharynx, turnover of mucus is more difficult and takes longer. Respiratory cilia of the lower airways can beat at a rate of 1,000 to 1,500 cycles per minute to propel mucus at progressively faster rates up the airways from 0.5-1 mm per minute to 5-20 mm per minute for small and large airways respectively (Harada and Repine, 1985). Consequently, it takes up to several hours to remove unwanted material from the distal airways.

Alongside the barrier function of mucus, the cellular component of the respiratory immune system also mitigates insult from infectious agents. Particularly in the lower respiratory tract, local immune populations activate and respond to inhaled stimuli that contact the epithelia or are detected in the mucosal lumen. These include alveolar monocytes and macrophages, as well as dendritic cells, $CD4^+$ T cells, and innate lymphoid cells that further clear debris from the lower airways (Janeway et al., 2008). Coughing is an additional mechanism by which respiratory secretions can be transported from the lower to the upper airways.

1.2 The Respiratory Microbiota

In healthy individuals, the thick, nutrient-rich mucus of the upper airways has been found to support approximately 10^6 bacteria/mL of oral wash (Charlson et al., 2011). However, microbial colonisation of the lower airways is prevented by the mucosal immune system. Although microbes have been consistently detected in the lower airways of healthy individuals (Charlson et al., 2011; Dickson et al., 2015; Erb-Downward et al., 2011), this likely represents an ongoing process of microaspiration (from the upper airways and external environment) and clearance (Figure 1.1). This model of a transient lower airway microbiota is supported by the similarity in microbiota composition between the upper and distal airways in healthy individuals (Charlson et al., 2011; Venkataraman et al., 2015).

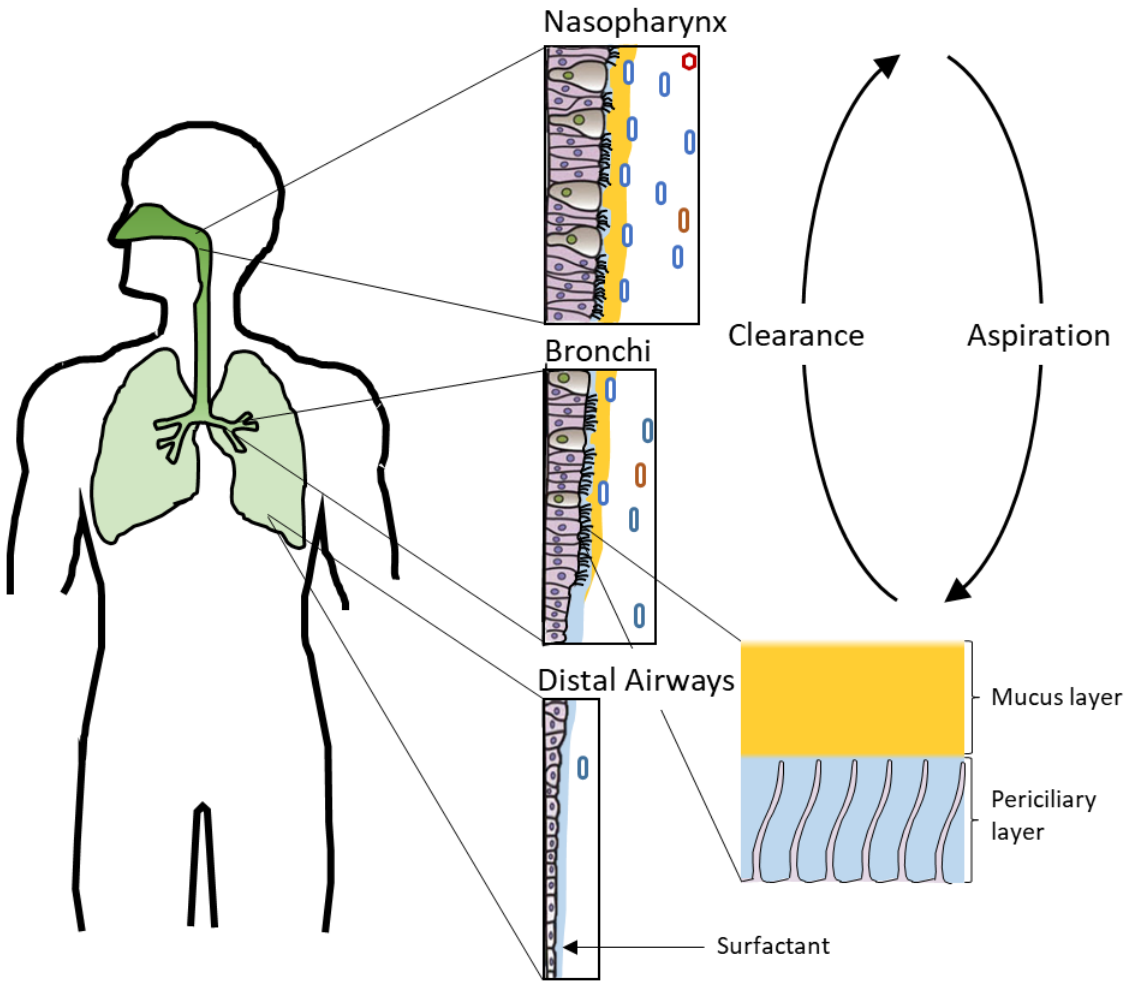

Figure 1.1: Respiratory mucosal surfaces in health, highlighting biogeographic diversity in epithelia, mucus thickness, and microbial colonisation at each site.

Disruption to the clearance mechanisms of the lower airways, however, provides an opportunity for microbes to grow in the lower airway environment and to persist, which is evident in patients with chronic airway diseases (Fahy and Dickey, 2010). The resulting inflammation and mucus secretion from microbial stimulation can further impair clearance (Roy et al., 2014; Segal et al., 2014). As well as this, the type and degree of airway inflammation and mucus secretion act as selective pressures on the composition of the microbiota. (Dicker et al., 2018; Ehre et al., 2012; Li et al., 1997). Therefore, respiratory factors such as inflammation and mucus secretion are strong determinants of the lower airway microbiota composition (Rogers et al., 2013a). Further, prescribed antibiotics and anti-

inflammatories that target pathogen infection and host immunity (Chung et al., 2014; Wang et al., 2010) can also have indirect effects on host-microbe interactions (Altenburg et al., 2011; Waterer et al., 2011). These drugs have therefore been found to influence the composition of the lower airway microbiota (Daniels et al., 2013; Goleva et al., 2013). Figure 1.2 summarises the described interactions, which are central to this dissertation. By understanding these interactions (e.g. how particular drugs and host factors influence the microbiota, and vice versa) we can use microbiota data to gain a deeper understanding of patients' lower airway environment (Rogers et al., 2013a). While acknowledging that chronic airway diseases are complex and multifactorial, by exploring the lower airway interactions described in Figure 1.2 and the clinical associations of these interactions, we can assess whether measuring the airway microbiota can contribute to a more effective and precise model of airway disease management. This dissertation aims to characterise the selective pressures of airway inflammation, mucus secretion, and drug treatments on the airway microbiota, and to assess the clinical implications of these interactions. The specific interactions explored in this dissertation relate to the chronic airway diseases: asthma and bronchiectasis, to which we now turn our attention.

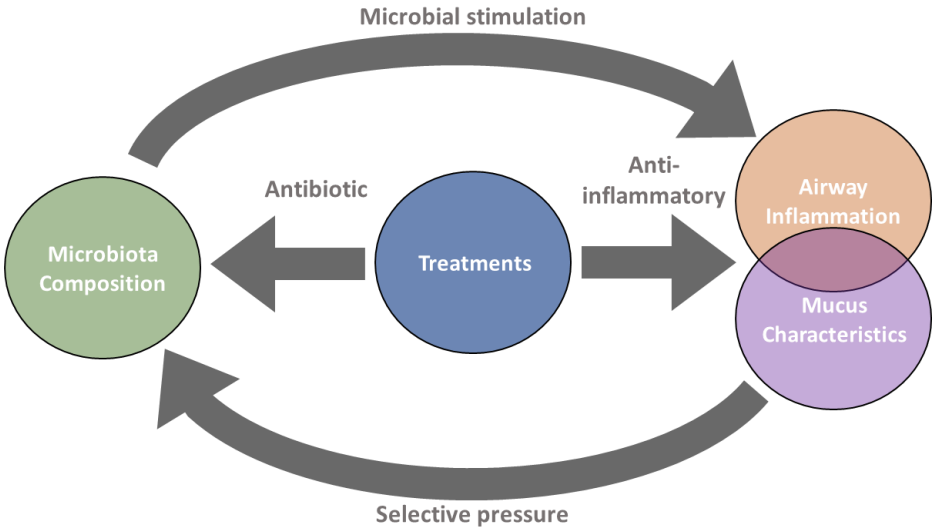

Figure 1.2: The central determinants of the lower airway environment in patients with chronic airway diseases

1.3 The Lower Airway Microbiota in Asthma

Asthma is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity with variable airflow limitation (Global Initiative for Asthma, 2017), affecting an estimated 235 million people (World Health Organisation, 2017). Severity of asthma varies greatly within this population, with an estimated 5-10% patients defined as having severe refractory asthma (Chung et al., 2014). These patients experience more severe airway inflammation (Pavord et al., 1999), have more mucus in the central and peripheral airways (Aikawa et al., 1992), and disproportionately contribute to asthma-associated hospitalizations and healthcare costs (Calhoun et al., 2014; Ivanova et al., 2012; Wang et al., 2010).

The main factor that contributes to asthma is considered to be immune dysfunction. Dysfunction that involves improper, type 2 immune response towards allergens, resulting in eosinophilic airway inflammation is the most common and well understood (Pavord et al., 1999). Nonspecific anti-inflammatory drugs, such as inhaled corticosteroids (ICS) effectively manage overt immune responses in the majority of patients who display this inflammatory phenotype. However, a subpopulation of patients experience asthma symptoms in the absence of eosinophilic inflammation (Pavord et al., 1999; Simpson et al., 2006). While these patients with a non-eosinophilic phenotype occur across the spectrum of asthma severity (Green et al., 2014; Pavord et al., 1999; Wenzel, 2012), they typically respond poorly to corticosteroids and do have a normal subepithelial layer thickness (Berry et al., 2007), indicating a different disease physiology. Indeed, based on relative numbers of sputum eosinophils and neutrophils, four inflammatory subtypes have been described: eosinophilic asthma, neutrophilic asthma, mixed granulocytic asthma, and paucigranulocytic asthma (Simpson et al., 2006).

Owing to the variability of asthma severity, treatment regime, and immune phenotype, characterisation of the lower airway microbiota is difficult, as these can all affect microbiota

diversity. For example, the microbiota of severe asthma has been shown to feature high relative abundances of pathogenic taxa including *Moraxella catarrhalis, Haemophilus* or *Streptococcus*, with predominance associated with longer asthma disease duration and worse lung function (Green et al., 2014). In other severe asthma studies, bacterial composition was associated with body mass index and Asthma Control Questionnaire (ACQ) scores (Huang et al., 2015).

The response to ICS has also been investigated in asthma. Despite ICS being a primary treatment option, a subset of asthma patients are ICS-resistant (Martin et al., 2007). A study that assessed the airway microbiome in patients with either ICS-resistant or ICS-sensitive asthma reported that the composition of the microbiome differed between the two groups, with the ICS-resistant samples featuring increased abundance of *Neisseria*, *Haemophilus*, *Simonsiella*, *Campylobacter*, *Leptotrichia*, *Tropheryma*, *Leuconostoc* and *Megasphaera* (Goleva et al., 2013). Furthermore, *in vitro* culture of primary human monocytes with these bacteria resulted in inhibited corticosteroid responses. These findings suggest that the airway microbiota might influence the efficacy of corticosteroid treatment. Supporting this, another study found oral corticosteroid use was an important factor affecting the relative abundance of the taxa that were significantly enriched in asthmatic patients (Denner et al., 2016).

Regarding immune phenotype, seemingly conflicting associations have been reported. In steroid-free patients, the airway microbial composition was associated with airway eosinophilia and airway hyperresponsiveness to mannitol but not airway neutrophilia (Sverrild et al., 2017). However, in patients with poorly controlled asthma, neutrophilia was associated with reduced airway bacterial diversity (Simpson et al., 2016). As immune phenotype is linked with both asthma severity (Green et al., 2014; Pavord et al., 1999; Wenzel, 2012) and response to treatment (Berry et al., 2007), the direct effect of immune phenotype on the microbiota is difficult to assess.

In Chapter 2, the association between immune phenotype and lower airway microbiota composition in asthma is assessed using sputum samples obtained from adult patients with persistent uncontrolled asthma, recruited as part of the Asthma and Macrolides: The Azithromycin Efficacy and Safety (AMAZES) study (Gibson et al., 2017). These patients had a history and documented objective evidence of variable airflow obstruction from bronchodilator response (with post-bronchodilator reversibility of at least 12% and at least 200 mL forced expiratory volume in 1 second (FEV_1)), airway hyperresponsiveness, or increased peak flow variability (>12% of amplitude above the lowest peak expiratory flow over at least 1 week of monitoring); and were currently symptomatic with at least partial loss of asthma control (asthma control score (ACQ6) ≥0.75) despite treatment with maintenance inhaled corticosteroids or long-acting bronchodilators. In this dissertation a second disease, bronchiectasis, is examined, which is now summarised.

1.4 The Lower Airway Microbiota in Bronchiectasis

Bronchiectasis is diagnosed by irreversible bronchial dilation, where either the internal diameter of the bronchus is larger than that of its accompanying vessel, or the bronchus fails to taper in the periphery of the chest (McGuinness and Naidich, 2002). Further, patients experience chronic or recurrent productive cough accompanied by frequent exacerbations of pulmonary symptoms. Global disease prevalence has been difficult to estimate (Chalmers et al., 2017), however rates of diagnosis have been shown to increase in the U.S.A (Weycker et al., 2017) and the U.K. (Quint et al., 2016). Accompanying bronchial dilation, patients also exhibit neutrophilic airway inflammation, mucus build-up, and chronic microbial infection. A proposed model for these bronchiectasis symptoms and pathophysiology is the vicious cycle model (Cole, 1986), which states that impaired mucus clearance, as a result of bronchiectasis, facilitates infection in the lower airways, causing host-mediated inflammatory response and

tissue damage, further driving airway remodelling and perpetuating irreversible bronchial dilation.

As described in this vicious cycle model, colonisation by pathogenic microbes is a result of lower airway mucus build-up. While *Haemophilus influenzae* and *Pseudomonas aeruginosa* are particularly common, and the most likely species to establish long-term infection (Cummings et al., 2010), the isolation of *Streptococcus pneumoniae*, *M. catarrhalis*, *Staphylococcus aureus*, and non-tuberculous mycobacteria (NTM) is also widespread (Foweraker and Wat, 2011). Assessment of the lower airway microbiota composition has identified taxa common across patients and typically abundant. This core airway microbiota included both the recognised airway pathogens *P. aeruginosa, H. influenzae* and *S. pneumoniae*, and members of *Veillonella*, *Prevotella* and *Neisseria* genera (Rogers et al., 2013b). In these patients, the diversity of the lower airway microbiota was found to correlate positively with lung function (Rogers et al., 2013b).

A number of studies have also found that patients fall into one of three broad airway microbiota types: those where *P. aeruginosa* is numerically dominant, those where *H. influenzae* is dominant, and those in which neither of these two species predominates (Cox et al., 2017; Rogers et al., 2015; Rogers et al., 2014b). The former type, with predominant *P. aeruginosa* infection, have been found to have poorer lung function (Ho et al., 1998), and more frequent exacerbations (Rogers et al., 2014b) and hospital visits (Loebinger et al., 2009). High antibiotic burden and frequent exacerbations have been suggested to be strong determinants of predominance of *P. aeruginosa* in the lower airways (Garcia-Nunez et al., 2014; Zhao et al., 2012), however whether other predictors exist is unknown.

Chapter 3 explores host genetic effects on the lower airway microbiota composition. Specifically, how a common host genetic variant (related to mucus characteristics) affects

lower airway infection in bronchiectasis. This was assessed using samples obtained from adults with bronchiectasis recruited as part of the Bronchiectasis and Low-dose Erythromycin (BLESS) study (Serisier et al., 2013). These patients had bronchiectasis, documented by high-resolution computed tomographic scan, at least two separate pulmonary exacerbations requiring supplemental systemic antibiotic therapy in the preceding 12 months, and daily sputum production. Patients did not have CF, current mycobacterial disease or bronchopulmonary aspergillosis, any reversible cause for exacerbations, maintenance oral antibiotic prophylaxis, prior macrolide use except short-term, changes to medications in the preceding four weeks, cigarette smoking within six months, and medications or comorbidities with the potential for important interactions with erythromycin. The specific genetic mutations of host mucous glycosyltransferases that are explored in Chapter 3 will now be discussed in more detail.

1.5 Mucosal Glycans and The Respiratory Microbiota

Throughout this introduction, mucus has been described as a physical barrier protecting the respiratory tract and its build-up in the lower airways highlighted as an important factor promoting lower airway microbial infection and chronic airway disease persistence. Therefore, the components that constitute mucus and are responsible for mucous functions may inform lower airway microbial infection in chronic airway disease.

The molecular composition of mucus consists of intricate glycan structures on secreted proteins and lipids (McGuckin et al., 2011; Thornton et al., 2008). This glycosylation process is mediated by a diverse family of glycosyltransferase enzymes expressed within epithelial cells (Delmotte et al., 2002). In patients with chronic airway diseases, airway inflammation can alter the expression of glycosyltransferases, changing the glycans displayed on the respiratory mucosal surface (Davril et al., 1999; Schulz et al., 2007). For example, patients with chronic

bronchitis and CF were found to exhibit altered sialylation, fucosylation, and sulfonation of sputum mucin proteins (Rose and Voynow, 2006). Bacteria utilize glycans for adherence, nutrients and gene regulators (McGuckin et al., 2011) and are therefore affected by mucin glycosylation. For example, *P. aeruginosa* preferentially binds to highly sialylated mucin glycans (Scharfman et al., 1999), and hence adheres to CF mucin over non-CF mucin (Devaraj et al., 1994).

The display of mucosal glycans is also dependent on functional genetic copies of glycosyltransferase genes. Inherited mutations in glycosyltransferase genes are common throughout the population and have been shown to affect the susceptibility to a variety of mucosal infections and chronic diseases (Cooling, 2015; Dotz and Wuhrer, 2016; Ferrer-Admetlla et al., 2009; McGuckin et al., 2011). Of the common mutations to glycosyltransferase genes, those in the α(1,2)-fucosyltransferase gene, *FUT2*, pertain to this dissertation.

FUT2 is expressed in mucosal tissues, by a range of secretory epithelial cell types. The translated α(1,2)-fucosyltransferase enzyme facilitates attachment of the L-fucose monosaccharide to the terminal galactose on *O*-linked glycan chains, producing α(1,2)-fucosylated glycans (Cooling, 2015; Dotz and Wuhrer, 2016). This resulting α(1,2)-fucosylated glycan on mucosal surfaces is a highly versatile structure and can be further modified to form one of a number of clinically important glycans, including the A and B histo-blood group antigens (Cooling, 2015; Dotz and Wuhrer, 2016). Mucosal histo-blood groups are analogous to those found on erythrocytes, although only those secreted by mucosal surfaces are dependent on *FUT2*. For example, an individual who has an A blood type will express A-type glycans on erythrocytes, but will only express A-type glycans on mucosal surfaces if they have a functional *FUT2* (Cooling, 2015). After the glycosylation process, α(1,2)-fucosylated proteins and lipids are either secreted from mucosal epithelium into the lumen directly, or are anchored to the apical cell surface membrane. Because *FUT2* controls the nature of the various α(1,2)-

fucosylated glycans secreted by mucosal surfaces, it was termed the "*secretor*" gene, although it does not regulate secretion *per se* (Cooling, 2015).

1.5.1 *FUT2* and infection and disease susceptibility

A high frequency of nonsense single nucleotide polymorphisms (SNPs) exists within the *FUT2* gene in humans (Genomes Project et al., 2015). Approximately one fifth of the global population harbour two non-functional alleles and are therefore unable to express α(1,2)-fucosylated glycans on mucosal surfaces (Ferrer-Admetlla et al., 2009; Genomes Project et al., 2015). While individuals with a functional *FUT2* allele are termed "secretors", those with loss-of-function mutations are termed "non-secretors".

Multiple SNPs are found in the *FUT2* coding region that confer loss, or hindered function, and the frequency varies with ethnicity (Ferrer-Admetlla et al., 2009; Genomes Project et al., 2015). The most common nonsense SNP in Caucasian, African, and central Asian populations is a G→A substitution at base pair 428 (rs601338), however the most common in east Asian populations is an A→T substitution at base pair 385 (rs1047781) (Ferrer-Admetlla et al., 2009; Genomes Project et al., 2015). Both SNPs occur at similar frequencies in their respective populations with estimates dating the emergence of the 428G→A mutation to at least 1.87 million years ago and the 385A→T mutation to at least 256,000 years ago (Silva et al., 2010). The age and frequency of these mutations suggest they are maintained in the genepool by balancing selection, where both secretor and non-secretor variants provide selective advantage. Population level genetic frequency studies suggest that the driver of this balancing selection is differential resistance to infection (Fumagalli et al., 2009), although identification of the causative infective agent is speculative. However, this phenomenon has been observed outside of the human population, where infection driven selection of glycosyltransferase variants was reported in a study of rabbit populations, where those with

endemic rabbit haemorrhagic disease virus had glycosyltransferase SNPs at higher frequencies compared to populations without the endemic virus (Guillon et al., 2009).

A clue as to why these loss-of-function mutations are carried at such a high frequency, and an illustration more generally of the importance of surface glycans to infection susceptibility, is the major differences in rates of bacterial- and viral-mediated diseases between secretors and non-secretors (Table 1.1 and Table 1.2). A large number of studies have now reported significantly higher rates of viral infection in secretors, including life-threatening infections caused by Human Immunodeficiency Virus (HIV), influenza, and norovirus (Ali et al., 2000; Chanzu et al., 2015; Currier et al., 2015; Kindberg et al., 2007; Kindberg et al., 2006; Raza et al., 1991). At the same time, secretors appear to be at a reduced risk of infections caused by bacterial pathogens, including *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*, and *Salmonella enterica* serovar Typhimurium (Blackwell et al., 1986a; Blackwell et al., 1986b; Boren et al., 1993; Goto et al., 2014; Pickard et al., 2014). This dichotomy in susceptibility also extends to chronic multifactorial diseases, such as chronic pancreatitis (Weiss et al., 2015), and diseases of altered immune regulation, such as asthma (Kauffmann et al., 1996; Ronchetti et al., 2001), type 1 diabetes (Smyth et al., 2011), and psoriasis (Tang et al., 2014; Wei et al., 2018).

Table 1.1: Secretor status and susceptibility to infections

Infection	Secretors more susceptible	Non-secretors more susceptible	Reference
Norovirus (GII.4)	✓		(Currier et al., 2015; Lopman et al., 2015)
Rotavirus (VP8)	✓		(Hu et al., 2012; Huang et al., 2012; Imbert-Marcille et al., 2014)
Influenza A virus	✓		(Raza et al., 1991)
Rhinovirus	✓		(Raza et al., 1991)
Echovirus	✓		(Raza et al., 1991)
Respiratory syncytial virus	✓		(Raza et al., 1991)
Human Immunodeficiency Virus	✓		(Ali et al., 2000; Chanzu et al., 2015; Kindberg et al., 2006)
Helicobacter pylori	✓		(Azevedo et al., 2008; Boren et al., 1993; Magalhaes et al., 2009)
Mumps		✓	(Azad et al., 2018; Tian et al., 2017)
Candida albicans		✓	(Hurd and Domino, 2004; Thom et al., 1989)
Streptococcus pneumoniae		✓	(Blackwell et al., 1986a)
Neisseria meningitidis		✓	(Blackwell et al., 1986a)
Haemophilus influenzae		✓	(Blackwell et al., 1986b)
Salmonella enterica serovar Typhimurium*		✓	(Goto et al., 2014)
*Citrobacter rodentium**		✓	(Pickard et al., 2014)
Campylobacter jejuni		✓	(Morrow et al., 2004; Ruiz-Palacios et al., 2003)
Urinary tract infection		✓	(Kinane et al., 1982; Sheinfeld et al., 1989)
Bacteraemia (after hematopoietic stem cell transplantation)		✓	(Rayes et al., 2016)

*Demonstrated in *Fut2*$^{-/-}$ mice with no human epidemiological evidence

Table 1.2: Secretor status and susceptibility to diseases

Disease	Secretors more susceptible	Non-secretors more susceptible	Reference
Asthma severity	✓		(Innes et al., 2011)
Graft-versus-host disease	✓		(Rayes et al., 2016)
Intestinal-type gastric cancer		✓	(Duell et al., 2015)
Primary sclerosing cholangitis		✓	(Folseraas et al., 2012)
Crohn's disease		✓	(Franke et al., 2010; McGovern et al., 2010)
Coeliac disease		✓	(Parmar et al., 2012)
Asthma		✓	(Kauffmann et al., 1996; Ronchetti et al., 2001)
Type 1 diabetes		✓	(Smyth et al., 2011)
High plasma vitamin B12		✓	(Hazra et al., 2008; Tanaka et al.; Tanwar et al., 2013)
Chronic Pancreatitis		✓	(Weiss et al., 2015)
Psoriasis		✓	(Tang et al., 2014; Wei et al., 2018)
Acute Uncomplicated Pyelonephritis		✓	(Ishitoya et al., 2002)
Behçet's disease		✓	(Xavier et al., 2015)

Despite the well-described associations between *FUT2* and a diversity of infections and diseases, our understanding of the mechanisms behind these relationships remains poor. Infection and pathogenesis are complex processes, with mucosal glycans likely influencing susceptibility through both direct and indirect mechanisms.

1.5.2 *FUT2* and pathogen adherence

α(1,2)-fucosylated glycans influence infection susceptibility directly, through facilitating pathogen adherence. As has been reviewed in detail (Audfray et al., 2013; Goto et al., 2016; Pickard and Chervonsky, 2015), multiple bacteria encode specific receptors that bind to host α(1,2)-fucosylated glycans for pathogen adherence. A well characterised example of this is in *Helicobacter pylori*, facilitated by the BabA adhesin. BabA has a specificity for the "Lewis b" α(1,2)-fucosylated mucosal glycan, therefore BabA expressing *H. pylori* are more

readily able to adhere to the gastric mucosa and colonize the stomachs of secretor individuals (Ilver et al., 1998). *BabA*-encoding *H. pylori* and subsequent infection susceptibility is something of an exception, as this is the only bacterial species listed in Table 1.1 where susceptibility is increased in secretors (due to the specificity of BabA towards α(1,2)-fucosylated glycans).

More complex *FUT2*-dependent pathogen adherence pathways have also been characterised, based on glycan location. Glycosylated proteins and lipids are abundant in the gastrointestinal tract, either anchored to the cell-surface, secreted into the lumen, or taken in through ingestion. Therefore, infection susceptibility, where pathogens adhere to glycans, depends on the location and anchoring of the glycan. Glycans that are not attached to the epithelium can, in fact, reduce infection susceptibility by acting as receptor decoys. For example, the cell surface mucin, MUC1, carries Lewis-b glycans and is shed from the surface of gastric epithelial cells acting as a releasable decoy to limit adhesion by BabA-expressing *H. pylori* to other cell surface Lewis-b-expressing molecules (Linden et al., 2009). As a separate example, maternal secretor status affects milk glycosylation (Bode, 2012), which in turn affects infants' susceptibility to *Campylobacter jejuni* diarrhoea (Morrow et al., 2004). This has been attributed to *C. jejuni* binding to α(1,2)-fucosylated milk glycans (Ruiz-Palacios et al., 2003), which act as a receptor decoy in the infant, sequestering pathogens away from the epithelium. As these examples demonstrate, the dynamics of how glycan-mediated adherence (either membrane-bound or luminal) ultimately confer susceptibility or resistance to infection is complex.

1.5.3 *FUT2* and the microbiota

In addition to influencing pathogen adherence, *FUT2* has been shown to also affect infection susceptibility indirectly. For example, mouse studies have shown that presence of

Fut2 reduces susceptibility to *S. Typhimurium*, *Enterococcus faecalis* and *Citrobacter rodentium* infection through the effect of α(1,2)-fucosylated glycans on the commensal gut microbiota (Goto et al., 2014; Pham et al., 2014; Pickard et al., 2014). Even small changes to microbiota composition can alter nutrient availability, profoundly affecting the ability of bacterial pathogens to colonise the gut (Servin, 2004). Beyond such "colonisation resistance", mice studies have also shown that *Fut2*-dependent fucosylated glycans are an important endogenous nutrient for commensal microbes, facilitating rapid host recovery following periods of stress caused by intestinal infection or inflammation (Pickard et al., 2014). These findings are supported by *in silico* analyses of microbial structure stability using microbiota data from mouse *Fut2* knock-out studies (Rausch et al., 2017).

In contrast, two large studies in healthy adult humans reported no difference in faecal microbiota composition between secretors and non-secretors (Davenport et al., 2016; Turpin et al., 2018), contradicting previous, positive associations from a smaller cohort (Wacklin et al., 2011). However, the use of intestinal mucosal biopsies in the latter study, where a greater host genotype effect may be expected (Spor et al., 2011), may explain this discrepancy (Rausch et al., 2017).

Differences in microbiota composition and resilience may also explain the numerous diseases associated with secretor status (as detailed in Table 1.2). Many of these conditions (including asthma, Crohn's disease, coeliac disease and psoriasis) are associated with intestinal microbiota composition (Cho and Blaser, 2012; Manichanh et al., 2006; Sanz, 2015). If secretor status can influence gut microbiology, it is reasonable to suggest that secretor status may contribute to microbiota-related disease susceptibility among predisposed individuals, as discussed elsewhere (Dotz and Wuhrer, 2016; Goto et al., 2016; McGovern et al., 2010; Pickard and Chervonsky, 2015; Rausch et al., 2011; Tong et al., 2014; Wacklin et al., 2011). However, given the numerous confounding environmental exposures in human populations, large cohort

studies with detailed metadata are required to determine the contribution of secretor status to these complex, multifactorial diseases. In Chapter 3, analysis of secretor status in the BLESS cohort of patients with bronchiectasis is presented. The effect of genetic mutations to *FUT2* on airway pathogen infection, airway microbiota composition and clinical characteristics of bronchiectasis severity was assessed. The final selective pressure covered in this dissertation relates to the effect of pharmaceuticals (specifically macrolides) on the airway microbiology.

1.6 Managing Chronic Airway Diseases with Macrolides

As depicted earlier in Figure 1.2, characteristic to chronic airway diseases are altered inflammation, mucus secretion, and airway infection. Most pharmaceuticals used to treat patients with chronic airway diseases act on one or more of these targets, including macrolides. Macrolides are drugs with a macrocyclic lactone ring of 12 or more elements (Mazzei et al., 1993). Of these, the 14- and 15-membered macrolides are commonly used in respiratory medicine (Altenburg et al., 2011). Several randomised controlled trials have demonstrated that administration of macrolides (erythromycin or azithromycin) over a prolonged period can effectively reduce exacerbations and improve quality of life in patients with bronchiectasis (Altenburg et al., 2013; Serisier et al., 2013; Wong et al., 2012) and recently, azithromycin has been shown to reduce exacerbations and improve quality of life in patients with persistent uncontrolled asthma (Gibson et al., 2017). However, macrolides have been found to affect a range of host functions as well as microbial functions, making it difficult to identify the mechanism of action in chronic airway disease management. The effects and clinical relevance to chronic airway disease are briefly summarised below.

1.6.1 Macrolides and mucus hypersecretion

Hypersecretion of mucus is a common feature of many chronic airway diseases and can facilitate airway obstruction and bacterial colonisation (Fahy and Dickey, 2010). Through *in*

vitro and *in vivo* studies, macrolides have been shown to modulate secretion of mucus. *In vitro,* erythromycin and clarithromycin have been shown to reduce mucus secretion in a dose dependent manner in primary respiratory epithelial cells (Goswami et al., 1990; Shimizu et al., 2003). In mice infected with *P. aeruginosa*, clarithromycin inhibited mucus production (Kaneko et al., 2003). In humans, clarithromycin has also been shown to improve the transportability of secretions in human subjects (Rubin et al., 1997; Tamaoki et al., 1995). In bronchiectasis, 8-week administration of erythromycin reduced sputum volume without significantly altering sputum bacterial density or inflammatory profiles (Tsang et al., 1999).

1.6.2 Macrolides and immune modulation

Macrolides have been shown to modulate multiple areas of host immunity including: suppressing inflammatory mediators, moderating leukocyte recruitment, and improving phagocytic function (Kanoh and Rubin, 2010). Neutrophilic airway inflammation is considered dependent on the release of IL-8, a potent neutrophil chemoattractant and erythromycin has been found to reduce IL-8 from subjects with chronic airway diseases (Oishi et al., 1994; Sakito et al., 1996), and atopy (Kohyama et al., 1999). This is supported by *in vitro* studies showing similar suppressive effects of macrolides on IL-8 production (Khair et al., 1995; Kikuchi et al., 2002).

Apart from suppressing overt IL-8 mediated inflammation, macrolides have also been shown to restore phagocytic function of macrophages. Alveolar macrophages are required for the effective clearance of apoptotic bronchial epithelial cells (efferocytosis) and certain bacterial species (phagocytosis) from the airways of patients with chronic airway diseases (Hodge et al., 2011; Hodge et al., 2003; Simpson et al., 2013; Ween et al., 2016). Uncleared material has been shown to have proinflammatory effects (Hodge et al., 2005). Azithromycin

has been previously shown to improve macrophage efferocytic (Hodge et al., 2006; Hodge et al., 2008) and phagocytic (Hodge and Reynolds, 2012) functions.

Modulation of anti-viral effects in epithelial cells have also been described. Azithromycin, but not erythromycin was found to significantly increase interferon response to rhinovirus in primary human bronchial epithelial cells (Gielen et al., 2010). Another *in vitro* study showed that erythromycin reduced rhinovirus infection of tracheal epithelial cells by suppression of ICAM-1, which is required for rhinovirus adherence (Suzuki et al., 2002). However, several randomised double-blind placebo-controlled trials have failed to show an effect of macrolides at reducing severity of infant viral-associated bronchiolitis (Kneyber et al., 2008; McCallum et al., 2015; Pinto et al., 2012). Multiple other studies have demonstrated that 14- and 15-membered macrolides elicit a range of immunomodulatory effects that would affect the airways of patients with chronic airway diseases. Only mechanisms relevant to this dissertation are mentioned here, however other immunomodulatory effects of macrolides are discussed elsewhere (Altenburg et al., 2011; Kanoh and Rubin, 2010).

1.6.3 Macrolides and the effects on bacteria

Macrolides have been shown to alter bacterial gene regulation and protein synthesis in patients with chronic lung disease and persistent bacterial infection. The former, bacterial gene regulation, is evidenced by studies demonstrating macrolides alter *P. aeruginosa* gene regulation. These studies have shown that macrolides reduce *P. aeruginosa* quorum sensing through the inhibition of *las*I and *rhl*I expression and deactivation of the autoinducer 3-oxo-C12-HSL (Nalca et al., 2006; Tateda et al., 2001). Quorum sensing controls many aspects of growth and behaviour of *P. aeruginosa*, including the production of elastase, pyocyanin, and exotoxin A (Imperi et al., 2014; Williams et al., 2000). In *P. aeruginosa*-infected CF mice,

azithromycin treatment resulted in a decreased bacterial load, neutrophil infiltration, and lung pathology, in part by blocking quorum sensing (Hoffmann et al., 2007).

Along with the quorum sensing regulatory effect of macrolides, they also have broad-spectrum bacteriostatic properties. Macrolides bind to the 23S ribosomal RNA of bacteria and inhibit protein synthesis (Hansen et al., 2002). For this function, macrolides are routinely prescribed to treat infections from nontuberculous mycobacterium (Egelund et al., 2015), and atypical bacteria in cases of community acquired pneumonia (Waterer et al., 2011) and sexually transmitted infections (Workowski et al., 2015).

However, owing to the antibiotic properties of macrolides, prolonged administration has been reported to increase the carriage of macrolide-resistant bacteria (Altenburg et al., 2013; Brusselle et al., 2013; Kastner and Guggenbichler, 2001; Malhotra-Kumar et al., 2007; Mustafa et al., 2017; Saiman et al., 2010; Serisier et al., 2013). While macrolide resistance has not been shown to directly alter the efficacy of macrolides to reduce exacerbations in patients (Altenburg et al., 2013), prolonged therapy might yet have deleterious wide-spread consequences. For example, in a post-hoc analysis of the BLESS trial in bronchiectasis, (Rogers et al., 2014a) low-dose erythromycin administered for 48-weeks was associated with altered sputum microbial composition if the baseline sputum was dominated by non *P. aeruginosa* organisms. In CF, no clinical benefit of azithromycin was found in patients after 12 months continuous treatment, instead, all *Staphylococcus aureus* strains became macrolide-resistant (Samson et al., 2016). Macrolide resistance can be acquired intrinsically, through genetic mutations, or extrinsically, through acquisition of transmissible macrolide resistance genes from a resistant organism. Acquired macrolide resistance is a global health concern, particularly relating to the treatment of the aforementioned infections where macrolides are routinely prescribed (Egelund et al., 2015; Waterer et al., 2011; Workowski et al., 2015). Identifying reservoirs of transmissible resistance genes is a global health strategy to limit the

dissemination of antibiotic resistance to the wider community (World Health Organisation, 2018). In Chapter 4, an undirected method is presented to assess changes to the carriage of antibiotic resistance genes in the BLESS cohort of bronchiectasis patients receiving erythromycin to reduce exacerbations. I sought to determine whether shotgun metagenomic sequencing, performed on a pooled sample, could be used to screen for resistance genes in large patient populations. This method was applied in Chapter 5 to assess changes to the carriage of antibiotic resistance genes in the AMAZES cohort where patients received azithromycin.

1.6.4 Macrolides and the lower airway microbiota

Through one or more of these mechanisms of action of macrolides, their use has been found to alter the composition of bacteria in the airways (Choo et al., 2018; Rogers et al., 2014a; Segal et al., 2017; Slater et al., 2014). In bronchiectasis, for example, long-term erythromycin treatment has been shown to reduce the relative abundance of *H. influenzae* and increase the relative abundance of the airway pathogen *P. aeruginosa* in the lungs (Rogers et al., 2014a). In asthma, analysis of five patients showed that six weeks of azithromycin reduced the airway microbiota diversity (Slater et al., 2014). In chronic obstructive pulmonary disease (COPD), analysis of 10 patients showed that eight weeks of azithromycin reduced airway microbiota diversity and reduced markers of airway inflammation (Segal et al., 2017). However, these studies do not adequately address the long-term microbiological effects of azithromycin in asthma. In Chapter 5, analysis of the effect of azithromycin to the airway microbiota in the AMAZES cohort of patients with persistent uncontrolled asthma is presented.

1.7 Dissertation Aims

As depicted in Figure 1.2, complex host-microbial interactions in the lower airways contribute to chronic airway disease persistence and severity. The objective of this dissertation

is to characterise how lower airway selective pressures contribute to airway microbiota composition. The selective pressure of airway inflammation is addressed in Chapter 2, where association between immune phenotype and lower airway microbiology is explored in baseline samples from the AMAZES cohort of patients with persistent symptomatic asthma. The selective pressure of mucus glycosylation type is addressed in Chapter 3, where association between FUT2 "secretor status" and lower airway infection, and microbiota composition are explored in the BLESS cohort of patients with bronchiectasis. Finally, the selective pressure of macrolide therapy is assessed in Chapter 4, through optimization of metagenomic strategies to measure changes to carriage of antibiotic resistance genes in the BLESS cohort. This method is then applied again in Chapter 5 to assess antibiotic resistance in the AMAZES cohort. Chapter 5 also assesses the effect of macrolide therapy on the microbiome composition. I hypothesise that selective pressures of the airway environment (inflammation, mucus glycosylation, drug intervention) have profound impacts on the airway microbiome, which in turn influence clinical markers of disease.

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CHAPTER 2: INFLAMMATORY PHENOTYPE AND LUNG MICROBIOTA IN SEVERE ASTHMA

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>
> The supplementary information has been included in Appendix 1

2.1 Abstract

Asthma pathophysiology and treatment responsiveness are predicted by inflammatory phenotype. However, the relationship between airway microbiology and asthma phenotype is poorly understood. **I aimed to characterise airway microbiota in patients with symptomatic stable asthma and relate composition to airway inflammatory phenotype and other phenotypic characteristics.**

The microbial composition of induced sputum specimens collected from adult patients screened for a multicentre randomized controlled trial was determined by 16S rRNA gene sequencing. Inflammatory phenotypes were defined by sputum neutrophil and eosinophil cell proportions. Microbiota were defined using alpha and beta diversity measures, and inter-phenotype differences identified using SIMilatiry of PERcentages (SIMPER), network analysis, and taxon fold change. Phenotypic predictors of airway microbiology were identified using multivariate linear regression.

Microbiota composition was determined in 167 participants, classified as eosinophilic (n=84), neutrophilic (n=14), paucigranulocytic (n=60), or mixed neutrophilic-eosinophilic (n=9) phenotypes of asthma. Airway microbiology was significantly less diverse (p=0.022) and

more dissimilar (p=0.005) in neutrophilic compared to eosinophilic participants. Sputum neutrophil proportion, but not eosinophil proportion, correlated significantly with these diversity measures (alpha-diversity: Spearman's r=-0.374, p<0.001, beta-diversity: r=0.238, p=0.002). Inter-phenotype differences were characterised by a greater frequency of pathogenic taxa at high relative abundance, and reduced *Streptococcus*, *Gemella* and *Porphyromonas* relative abundance in neutrophilic asthma. Multivariate regression confirmed sputum neutrophil proportion was the strongest predictor of microbiota composition.

Neutrophilic asthma is associated with airway microbiology that is significantly different to that in other inflammatory phenotypes, particularly eosinophilic asthma. Differences in microbiota composition may influence response to antimicrobial and steroid therapies, and risk of lung infection.

2.2 Introduction

Asthma phenotypes, based on characteristics of airway inflammation, are increasingly recognised as an important prognostic indicator for asthma severity and treatment responsiveness (Wenzel, 2012). In addition to an allergen-induced Th2-lymphocyte, IL-5 mediated, eosinophilic inflammatory response, asthma can also occur in the absence of eosinophilic inflammation (termed non-eosinophilic asthma) (Pavord et al., 1999; Simpson JL et al., 2006). Indeed, based on relative numbers of sputum eosinophils and neutrophils, four inflammatory subtypes: eosinophilic asthma, neutrophilic asthma, mixed granulocytic asthma, and paucigranulocytic asthma, have been described (Simpson et al., 2006).

Unlike the relatively well-defined mechanisms that result in eosinophilic airway inflammation, those leading to non-eosinophilic asthma, particularly neutrophilic asthma, remain relatively poorly understood (Pelaia et al., 2015). Further, while non-eosinophilic phenotypes occur across the spectrum of asthma severity (Green et al., 2014; Pavord et al.,

1999; Wenzel, 2012), they typically respond poorly to corticosteroids (Green et al., 2014; Pavord et al., 1999). Inflammatory phenotypes have also been shown to differ with respect to airway microbiology. Compared with other patients with asthma, those with neutrophilic asthma are more likely to have a potentially pathogenic organism identified, by either culture-based (Essilfie et al., 2012) or culture-independent approaches (Green et al., 2014), and have reduced airway bacterial diversity (Simpson et al., 2016b). Given that airway microbiota composition is associated with the degree of airway hyper-responsiveness among patients with sub-optimally controlled asthma (Huang et al., 2011), inter-phenotype differences in airway microbiology are likely to be clinically important.

The relationships between asthma inflammatory phenotypes and airway microbiology are likely to be complex and bi-directional. Asthma phenotypes represent immunological and physicochemical differences within the lower airways that are likely to be reflected, through their selective effect on microbial growth and airway clearance, in divergent lower airway microbiota (Rogers et al., 2013). Where these differences involve the increased abundance of particular respiratory pathogens, or a depletion of commensal populations, they could contribute substantially to the course of airway disease or risk of adverse treatment events, such as corticosteroid associated pneumonia (McKeever et al., 2013). On the other hand, the characteristics of airway microbiology, even in the absence of frank infection, could influence asthma inflammatory phenotype. Defining the relationships between inflammatory phenotype and lower airway microbiota would inform our understanding of asthma pathophysiology and could help to identify prognostic markers.

Several previous studies have reported differences in airway microbiology in eosinophilic and non-eosinophilic asthma, and the existence of significant relationships between this microbiota composition and clinical asthma measures (Denner et al., 2016; Green et al., 2014; Huang et al., 2015; Simpson et al., 2016a; Zhang et al., 2016). However, while

providing important insight, these studies have involved relatively small and heterogeneous patient cohorts. This study, based on participants enrolled into the Asthma and Macrolides: the Azithromycin Efficacy and Safety Study (AMAZES) trial (ACTRN12609000197235), was over three times the size of any study performed previously and focused on a well-defined population of patients with severe but stable asthma, the majority of whom were treated with inhaled corticosteroids.

Through the application of a systematic approach to microbiota characterisation, I aimed to assess whether asthma inflammatory phenotypes were associated with substantially different lower airway microbiology, to identify bacterial taxa that discriminate among inflammatory phenotypes, and to determine the contribution of patient and clinical characteristics to variation in the composition of the bacterial component of the microbiome.

2.3 Methods

2.3.1 Study population

For full methodology see supplementary methods of Appendix 1. Analysis was performed on samples collected as part of the baseline screening population from AMAZES clinical trial (ACTRN12609000197235). Participants were recruited from eight centres across Australia. Pre-defined inflammatory phenotype categories, based on sputum cell counts relative to patient age, were assigned as published previously and detailed in the Supplementary Methods (Brooks et al., 2013; Simpson et al., 2006). Briefly, neutrophilic phenotype was defined as ≥61% neutrophils (neutrophil% cut-off dependent on age), eosinophilic phenotype as ≥3% eosinophils, paucigranulocytic as ≤61% neutrophils and ≤3% eosinophils and mixed granulocytic was defined as ≥61% neutrophils and ≥3% eosinophils.

2.3.2 Induced sputum sample collection

Sputum induction with hypertonic saline (4.5%) was performed by trained personnel as described previously (Gibson et al., 1998). Sputum aliquots were stored at -80°C for DNA extraction or dispersed using dithiothreitol for sputum cell count assessment and inflammatory subtype determination (Simpson et al., 2014).

2.3.3 DNA extraction

DNA extraction was performed on sputum sample aliquots of approximately 100 µl. Following the addition of 300 µl of phosphate buffered saline, samples were vortexed for 10 seconds and placed on ice for 2 min. Bacterial cells were then pelleted by centrifugation at 13,000 x g for 10 min. Following removal of supernatant, 300 µl of Tris-EDTA solution (10 mM Tris-HCl, 1 mM EDTA; pH 8.0; Ambion, ThermoFisher Scientific, Victoria, Australia), 200 µg of silica: zirconium beads (1:1 of 0.1 mm and 1.0 mm; Biospec Products, Inc., OK, USA), and a single chrome bead (3.2 mm, Biospec Products, Inc., OK, USA) were added to the tube containing the cell pellet. Samples underwent bead-beating at 6.5 m/s for 60 sec in a FastPrep®-24 Instrument (MP Biomedicals, CA, USA). Homogenised sample was heated to 90 °C for 5 min, before being cooled on ice for 5 min. Lysozyme (ROCHE, ThermoFisher Scientific, Victoria, Australia) and lysostaphin (Sigma-Aldrich, MO, USA) were then added to a final concentration of 2 mg/mL and 0.1 mg/mL, respectively, and samples incubated at 37 °C for 1 hr. Proteinase K (Fermentas, ThermoFisher Scientific, Victoria, Australia) and sodium dodecyl sulphate (Sigma-Aldrich, MO, USA) were then added to a final concentration of 1.2 mg/mL and 1.5 %, w/v, respectively. Following incubation at 30 min at 56 °C, 40 µl of 5M sodium chloride and 450 µl of phenol:chloroform:isoamyl alcohol (25:24:1; saline buffered at pH8.0; Sigma-Aldrich, MO, USA) were added and samples vortexed for 30 sec. The aqueous-organic layers were separated by centrifugation at 13,000 x g for 10 min and 400 µl of the

aqueous layer was transferred to a new microfuge tube. DNA was recovered using an EZ-10 Spin column in accordance with manufacturer's instructions (Bio Basic, Inc., Ontario, Canada), following precipitation by the addition of 10 M ammonium acetate and 99% ethanol (Sigma Aldrich, MO, USA) in a 1:10 and 1:1 ratio with sample volume, respectively. DNA was eluted in 50 µl UltraPure DNase/RNase-free distilled water (Gibco, ThermoFisher Scientific, Victoria, Australia) and stored at -80 °C prior to analysis.

2.3.4 16S rRNA gene amplicon sequencing

The V1-3 hypervariable region of the bacterial 16S rRNA gene was amplified from sputum DNA using modified primers 27F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGRGTTTGATCMTGGCTCAG-3') and 519R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTNTTACNGCGGCKGCTG-3'), with Illumina adapter overhang sequences as indicated by underline. Amplicons were generated, cleaned, indexed and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html) with certain modifications. Briefly, an initial PCR reaction contained at least 12.5 ng of DNA, 5 µL of forward primer (1 µM), 5 µL of reverse primer (1 µM) and 12.5 µL of 2× KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) in a total volume of 25 µL. The PCR reaction was performed on a Veriti 96-well Thermal Cycler (Life Technologies) using the following program: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec and a final extension step at 72 °C for 5 min. Samples were multiplexed using a dual-index approach with the Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The final library was paired-end sequenced at 2 × 300 bp using a MiSeq Reagent Kit v3 on the Illumina

MiSeq platform. Sequencing was performed at the David R Gunn Genomics Facility, South Australian Health and Medical Research Institute.

2.3.5 16S rRNA gene qPCR

Approximate 16S rRNA gene copy number was assessed by quantitative PCR (qPCR) using the 16S rRNA universal primers B331F (5'-TCCTACGGGAGGCAGCAGT-3') and B797R (5'-GGACTACCAGGGTATCTAATCCTGTT-3') using Platinum SYBR Green (ThermoFisher scientific, Vic, Australia) as previously described (Nadkarni et al., 2002). Reactions were performed in duplicate and averages taken. Sample total bacterial copy number was calculated per µL of DNA eluate against a standard curve of a known bacterial copy number.

2.3.6 Sequence data processing

The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) software was used to analyse the 16S rRNA sequence generated from paired-end amplicon sequencing using bioinformatics pipeline as previously described (Jervis-Bardy et al., 2015). Briefly, barcoded forward and reverse sequencing reads were quality filtered and merged using Paired-End reAd mergeR (PEAR v0.9.6). Chimeras were detected and filtered from the paired-end reads using USEARCH (v6.1) against the 97% clustered representative sequences from the Greengenes database (v13.8). Operational taxonomic units (OTUs) were assigned to the reads using an open reference approach with UCLUST algorithm (v1.2.22q) against the SILVA database release 111 (July 2012) that was clustered at 97% identity. Spurious OTUs were then removed systematically using previous reports of common laboratory sequencing contaminants (Salter et al., 2014). A minimum subsampling depth of 1,732 reads was then selected for all samples.

Where taxa assignment failed to classify to the Family or Genus level, OTU reference sequences (accounting for >99% of OTU reads) were separately aligned using SILVA

Incremental Aligner (SINA) (https://www.arb-silva.de/) which uses SILVA, RDP, Greengenes, LTP and EMBL sequence collections. If the alignments identified taxa to a genus level, and at >99% similarity, they replaced the previous taxon assignment. This occurred for Streptococcus II which was previously incorrectly assigned as Clostridiales;Other;Other. Streptococcus I refers to the OTU cluster which was assigned as Streptococcus during initial assignment.

2.3.7 Diversity measurements and statistical analyses

Five alpha diversity (within-sample variance) indices were employed to test a variety of parameters of within-patient taxon distribution: Faith's phylogenetic diversity (where a higher value indicates a more phylogenetically diverse sample), Simpson's and Pielou's evenness indices (where a higher value indicates a more equitable distribution of taxa abundance), taxa richness (the total number of taxa detected), and Shannon-Weiner diversity (a measure incorporating both the number and equitability of detected taxa). Beta diversity (inter-sample variance) was determined by using two approaches: weighted UniFrac similarity (which accounts for phylogenetic distance) and square-root transformed Bray-Curtis similarity (based on the relative abundance of taxa alone). Bray-Curtis matrix was calculated based on sample-normalised, square root transformed relative taxon abundance. Principal coordinate analysis (PCoA) was used to visualize clustering of samples based on their similarity matrices with PCO1 and PCO2 coordinates and group centroids plotted using ggplot2 package of R statistical software. Distance from centroid was calculated as previously described, using PRIMER (Anderson et al., 2006). Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) on the beta-diversity matrices was used to test the null hypothesis of no difference amongst a priori-defined groups using PERMANOVA + add-on package for PRIMER. These a priori-defined groups were the four inflammatory phenotypes. The test was computed using unrestricted permutation of raw data with 9,999 random

permutations and at a significance level of 0.01. Alpha and beta diversity measures were calculated using either QIIME (v. 1.8.0) or Primer (v. 6, PRIMER E Ltd, Plymouth, UK).

Continuous data were tested for non-normality including skewness and kurtosis using the D'Agostino-Pearson omnibus test. The Kruskal-Wallis one-way ANOVA with Dunn's post hoc test was used for multiple comparisons of non-normally distributed data, Mann-Whitney U test for pairwise comparisons, chi-squared test for categorical data, and Spearman's test for correlations (GraphPad PRISM, v.7.01 GraphPad Software Inc., California, USA).

Multivariate linear regression was performed using Faith's phylogenetic diversity and UniFrac distance from centroid as two dependent variables reflecting aspects of diversity (SPSS v.23.0, IBM, Armonk, NY). Covariates were selected a priori and included in the model based on a significant correlation with either dependent variable. Confidence intervals were obtained by bootstrapping; resampling 1,000 times. Covariates were tested for collinearity using variance inflation factors.

2.3.8 Taxon dispersion

Variation in microbiota composition at the genus-level was assessed using multiple approaches. First, taxa that contributed to the overall variation between the asthma phenotypes were identified using SIMilarity of PERcentages (SIMPER) analysis in PRIMER. Subsequently, the abundance of the 13 highest ranked taxa (accounting for 50% of the dissimilarity between neutrophilic and eosinophilic groups) were used to generate a heatmap using ggplot2 package of R statistical software. Hierarchical clustering of the taxa was performed on Bray-Curtis dissimilarity and clustered using single linkage method. Dominance of *Haemophilus* and *Moraxella* was determined when the relative abundance of each taxa exceeded 40%. This cut-off was selected based to the distribution of the relative abundance, where a clear distinction between samples with >40% and <40% was evident, suggesting

overgrowth of these taxa. Second, strong taxon-taxon correlations were identified using SparCC, where absolute taxon abundances were bootstrapped 100 times to generate correlation p values (Friedman and Alm, 2012). Networks were then generated from selected correlations ($r \geq 0.25$ or $r \leq -0.25$) and p values ($p \leq 0.01$) using Cytoscape (version 3.4.0).

Two approaches were used to investigate the impact of pathogen overgrowth on microbiota composition. First, in samples where *Haemophilus* or *Moraxella* were the dominant taxon and represented > 40% of total reads, their relative abundance was adjusted to the mean value for the study cohort and the remaining relative abundance measures rescaled, as described previously (Rogers et al., 2015). For example, the microbiota composition of a sample with 50% relative abundance of *Haemophilus,* 20% Taxa A, 10% Taxa B, 5% Taxa C, 5% Taxa D, 5% Taxa E, and 5% Taxa F would be rescaled to remove Haemophilus. The rescaled composition would comprise of 40% Taxa A, 20% Taxa B, 10% Taxa C, 10% Taxa D, 10% Taxa E, and 10% Taxa F. PERMANOVA analyses were then performed on the rescaled data. Second, pairwise comparisons between neutrophilic and eosinophilic samples were performed using the phyloseq R package with DEseq2 extension, based on count data (Love et al., 2014; McMurdie and Holmes, 2013). P values were corrected using the Benjamini-Hochberg false discovery rate procedure and a corrected alpha value cut-off of <0.05 used for inclusion.

2.4 Results

2.4.1 Clinical characteristics and sequence data

Induced sputum samples were obtained from 187 participants. Of these, 13 were excluded due to poor sample quality. Of the 174 that underwent 16S rRNA gene amplicon sequencing, a further seven were excluded due to an insufficient sequence read depth. The remaining 167 subjects were classified as one of four inflammatory phenotypes: neutrophilic

(n=14), eosinophilic (n=84), paucigranulocytic (n=60), or mixed granulocytic (n=9) based on previously described sputum inflammatory cell count percentages (Brooks et al., 2013; Simpson et al., 2006). There was no significant difference in age, gender distribution, atopy, smoking history, ICS dose, GINA treatment step, or mean ACQ6 score between these phenotypic groups, as assessed using multiple comparison tests (Table 2.1). There were, however, significant differences in lung function, as assessed by both FEV_1 % predicted ($p=0.035$) and FEV_1/FVC% ($p=0.013$).

Following quality filtering and chimera removal, 16S rRNA gene amplicon sequencing resulted in a median read depth of 12,792 (q1, q3: 8060, 16595). Sequence data were sub-sampled to a uniform depth of 1,732 reads based on rarefaction curve asymptotes and Good's coverage values (Table E1.1 of Appendix 1). No significant differences in total bacterial burden were found between inflammatory phenotypes ($p=0.51$, Kruskal-Wallis test, see Figure E1.1 of Appendix 1).

2.4.2 Alpha diversity

Participants with neutrophilic asthma had significantly lower Faith's phylogenetic score ($p=0.022$) than participants with eosinophilic asthma, which resembled that of paucigranulocytic asthma (Figure 2.1A). Faith's phylogenetic diversity significantly correlated with the sputum neutrophil % ($r=-0.374$, $p<0.0001$, Figure 2.1B) but not with sputum eosinophil % ($r=0.146$, $p=0.060$, Figure 2.1C). Analysis with a range of alternative alpha diversity indices (taxa richness, Shannon-Wiener index, Simpson's index, and Pielou's evenness, see Figures E1.2 and E1.3 of Appendix 1), resulted in consistent findings in relation to phenotype, sputum neutrophil % and sputum eosinophil %. Together, these results demonstrate a significant relationship between airway microbiota composition and sputum neutrophilia, but not sputum eosinophilia.

Table 2.1: Clinical and inflammatory cell parameters of participants

	Neutrophilic	Eosinophilic	Paucigranulocytic	Mixed Granulocytic	p
N	14	84	60	9	
Age, mean (SD)	59.8 (13.9)	57.2 (15.2)	55.8 (13.8)	56.8 (17.8)	0.817
Male, n (%)	9 (64.3)	30 (35.7)	23 (38.3)	6 (66.7)	0.084
Atopic, n (%)	11 (78.6)	62 (76.5), n=81	52 (86.7)	6 (66.7)	0.305
Previous smoker, n (%)	6 (42.9)	32 (38.1)	17 (28.3)	4 (44.4)	0.491
Smoking pack years, median (q1,q3)	22.0 (18.6,27.5)	5.3 (1.2, 15.9)	5.0 (1.3, 30.0)	4.4 (0.9, 63.6)	0.379
Duration of asthma, years median (q1,q3)	33.8 (3.5, 48.3)	36.3 (19.7, 49.2)	33.7 (14.2, 54.4)	53.6 (32.6, 60.4)	0.205
FEV_1 % predicted, mean (SD)	70.3 (18.2)	70.0 (17.9)	78.7 (19.2)*	68.4 (17.3)	0.035
FVC % predicted, mean (SD)	81.4 (11.6)	83.6 (16.4)	85.1 (15.0)	82.9 (11.7)	0.854
FEV_1/FVC %, mean (SD)	65.8 (14.5)	64.9 (12.2)	71.3 (12.2)*	63.9 (12.7)	0.013
ACQ6, mean (SD)	2.1 (1.2)	1.9 (0.9)	1.6 (0.8)	1.5 (0.6)	0.210
GINA Treatment step					0.058
1	1 (7.1)	2 (2.5)	0 (0)	0 (0)	
2	0 (0)	0 (0)	0 (0)	0 (0)	
3	0 (0)	10 (12.4)	12 (20.3)	2 (22.2)	
4	11 (78.6)	67 (82.7)	47 (80.0)	7 (77.8)	
5	2 (14.3)	2 (2.5)	0 (0)	0 (0)	
ICS dose, µg, median (q1,q3)	2000 (1280,2000), n=13	1000 (800,2000), n=82	1000 (800,2000), n=59	1600 (1000, 2000)	0.254
Total cell count, x 10^6/mL (q1,q3)	9.8 (7.6, 12.7)* †	3.6 (1.9, 7.6)	3.74 (2.0, 7.6)	8.64 (5.22, 11.34)	<0.001
Viability, %	89.2 (73.5, 93.0)* †	69.8 (52.2, 80.7)	68.9 (55.7, 79.5)	90.0 (84.4, 93.7)* †	<0.001
Neutrophils, %	75.0 (68.80, 84.00)* †	27.13 (14.38, 41.00)	34.13 (12.63, 49.25)	77.00 (71.25, 77.50)* †	<0.001
Eosinophils, %	0.63 (0.50, 1.00)*	6.88 (3.63, 18.13)	0.25 (0.00, 1.00)*	6.75 (3.25, 12.25)# †	<0.001
Macrophages, %	20.75 (14.50, 30.25)* †	52.75 (38.25, 71.00)	53.34 (43.50, 75.63)	16.00 (15.50, 20.25) *†	<0.001
Lymphocytes, %	0.63 (0.25, 1.00)	1.13 (0.25, 2.25)	0.88 (0.25, 2.63)	0.25 (0.00, 0.38)* †	0.018
Columnar epithelial cells, %	0.25 (0.00, 1.25)* †	2.17 (0.75, 6.00)	4.13 (1.21, 8.50)	0.50 (0.25, 1.50) †	<0.001
Squamous cells, %	1.11 (0.50, 3.85)* †	5.99 (2.32,16.23)	6.18 (2.92, 11.31)	3.38 (0.25, 8.05)	0.002

ACQ6: asthma control questionnaire 6 question format, FEV_1: forced expiratory volume in 1 second, FVC: forced vital capacity, ICS: inhaled corticosteroids, SD: standard deviation, q1, q3: quartile 1, quartile 3. P values in last column describe variance across four phenotypes. *p<0.05 vs eosinophilic asthma, †p<0.05 vs. paucigranulocytic, #p<0.05 vs. neutrophilic asthma.

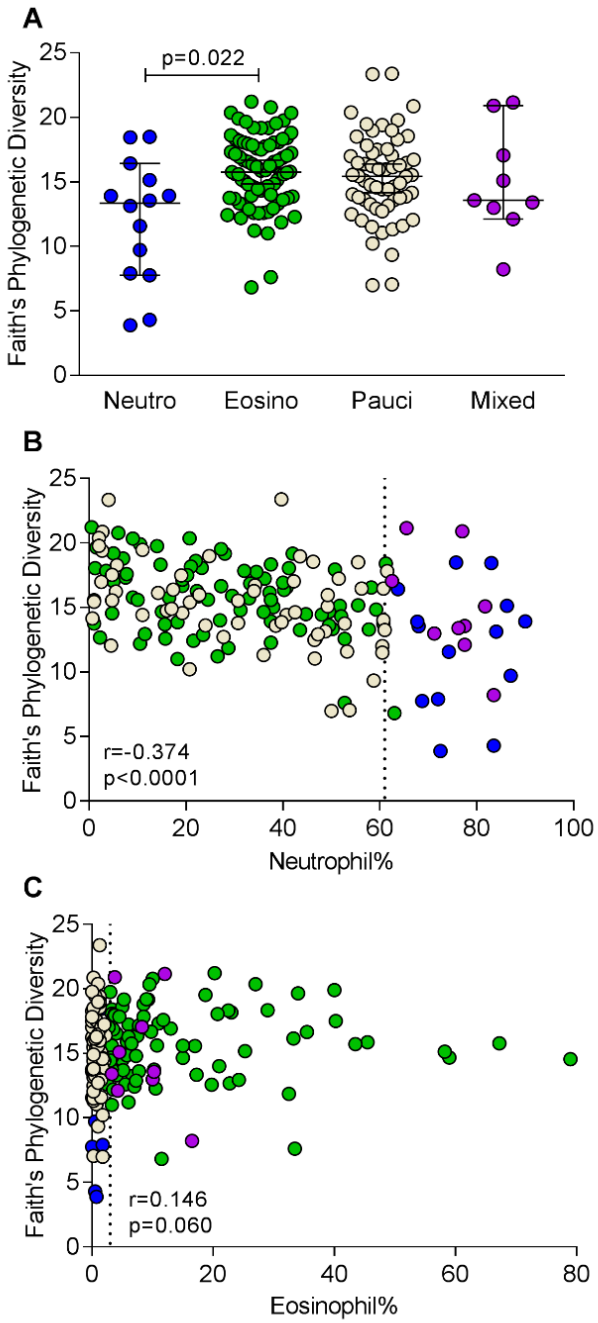

Figure 2.1: Faith's phylogenetic diversity is significantly associated with sputum neutrophilia but not eosinophilia. A) Patients grouped by asthma phenotype. B) Neutrophil % where dotted line at 61% neutrophils indicates phenotype cut-off point. C) Eosinophil % where dotted line at 3% eosinophils indicates phenotype cut-off point. Colours represent asthma phenotype, where blue= >61% neutrophils, green= >3% eosinophils, yellow= <61% neutrophils and <3% eosinophils (paucigranulocytic), and purple= both >61% neutrophils and >3% eosinophils (mixed). Statistical significance was assessed by A) Kruskal-Wallis one-way ANOVA with Dunn's post hoc test or B) and C) Spearman's rank correlation.

2.4.3 Beta diversity

Principal Coordinate Analysis (PCoA) of weighted UniFrac similarity distance showed that neutrophilic samples were distinguished from other phenotypes along the first and second principal coordinates, while the other phenotypes broadly clustered together (Figure 2.2A). Consistent with these observations, a PERMANOVA test showed that phenotype grouping contributed significantly to the differences in microbial composition of the samples ($p=0.0004$, pseudo-$F=3.997$, see Table E1.2 of Appendix 1). Pairwise PERMANOVA comparing the phenotype groups indicated that variance was attributed to neutrophilic vs eosinophilic ($p=0.0001$, $T=3.30$) and neutrophilic vs paucigranulocytic ($p=0.0015$, $T=2.52$) groups (see Table E1.3 of Appendix 1).

Assessment of microbiota dispersion based on distance from centroid was consistent with PERMANOVA analysis, with the samples from neutrophilic phenotype participants having significantly higher distances from the centroid than samples from eosinophilic and paucigranulocytic participants (Figure 2.2B). In keeping with alpha diversity analyses, variance in distance from centroid was associated with sputum neutrophil %, rather than sputum eosinophil % (see Figure E1.4 of Appendix 1). Beta-diversity analyses using a second distance measure, Bray-Curtis, gave consistent findings (see Figure E1.5 of Appendix 1).

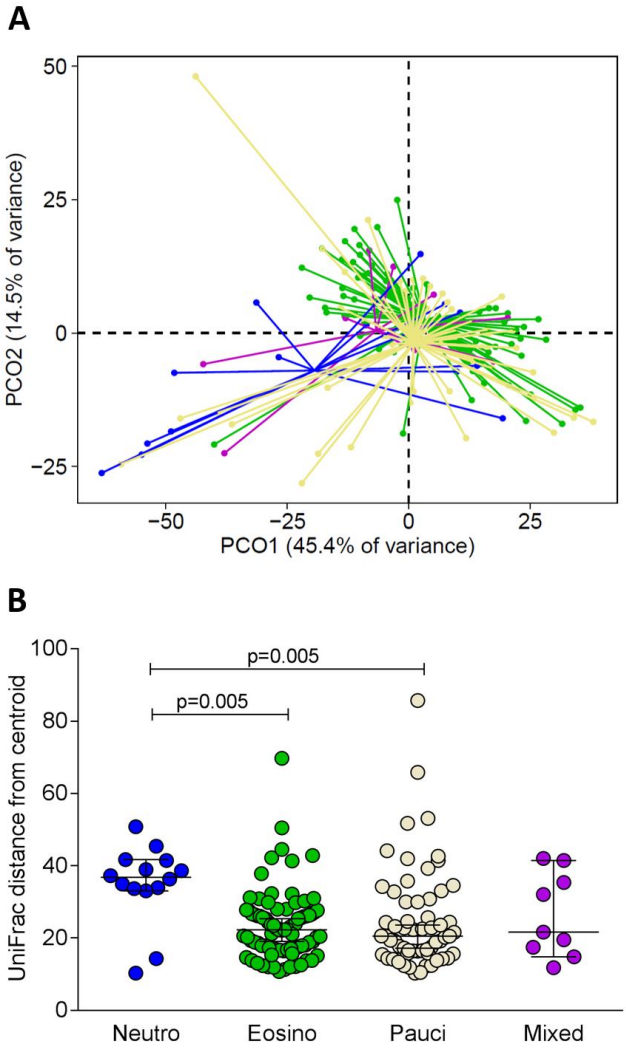

Figure 2.2: Microbiota dispersion grouped by asthma phenotype. A) Principal Coordinate Analysis (PCoA). The first two principal coordinates are plotted on the *x*- and *y*-axes, respectively (representing 59.9% of the total variation). B) Distance from centroid. Statistical significance was assessed by Kruskal-Wallis one-way ANOVA with Dunn's post hoc test.

2.4.4 Taxon distribution and network analysis

SIMPER analysis was used to rank taxa according to their contribution to intergroup variance in microbiota composition. Thirteen taxa were identified which cumulatively accounted for approximately 50% of total variance between neutrophilic and eosinophilic samples (see Table E1.4 of Appendix 1). Hierarchical cluster analysis based on relative taxon abundance revealed that *Moraxella* and *Haemophilus* clustered separately from the other eleven taxa (Figure 2.3). In the patients with neutrophilic asthma, *Moraxella* and *Haemophilus*

exceeded 40% relative abundance in 6/14 (42.9%) samples compared to only 1/84 (1.19%) patients with eosinophilic asthma, 7/60 (11.7%) with paucigranulocytic and 1/9 (11.1%) with mixed (χ^2=25.5, p<0.0001, Figure 2.3, Figure E1.6 of Appendix 1). Relationships between bacterial taxon relative abundance were further visualised by network analysis (Figure 2.4), revealing a bacterial community of taxa whose abundance is positively correlated in almost all cases. Most of these taxa were more prevalent in eosinophilic samples than in neutrophilic samples. *Haemophilus*, which had a mean abundance that was higher in neutrophilic samples, was the single exception, negatively correlating with other members of the sputum bacterial community.

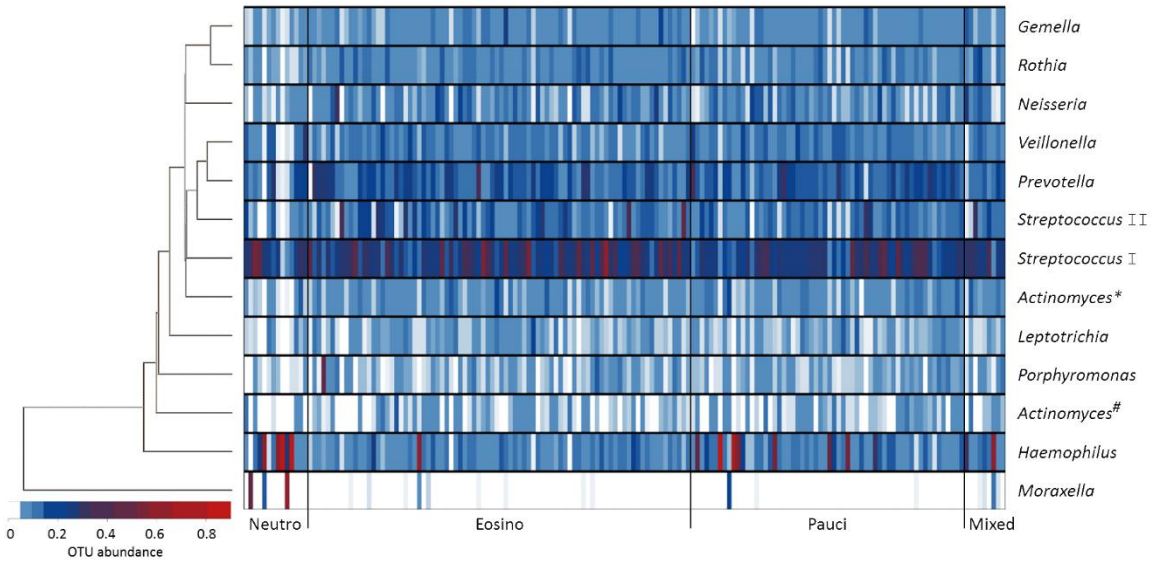

Figure 2.3: Relative abundance of discriminant taxa among asthma phenotypes. The 13 taxa that collectively contribute to approximately 50% of variance among phenotypes, as determined by SIMPER analysis. The clustering shows the similarity relationship of genera based on Bray-Curtis similarity distance and single linkage hierarchical clustering method. **Actinomyces* sp. uncultured bacteria, *#Actinomyces* sp. oral clone DR002.

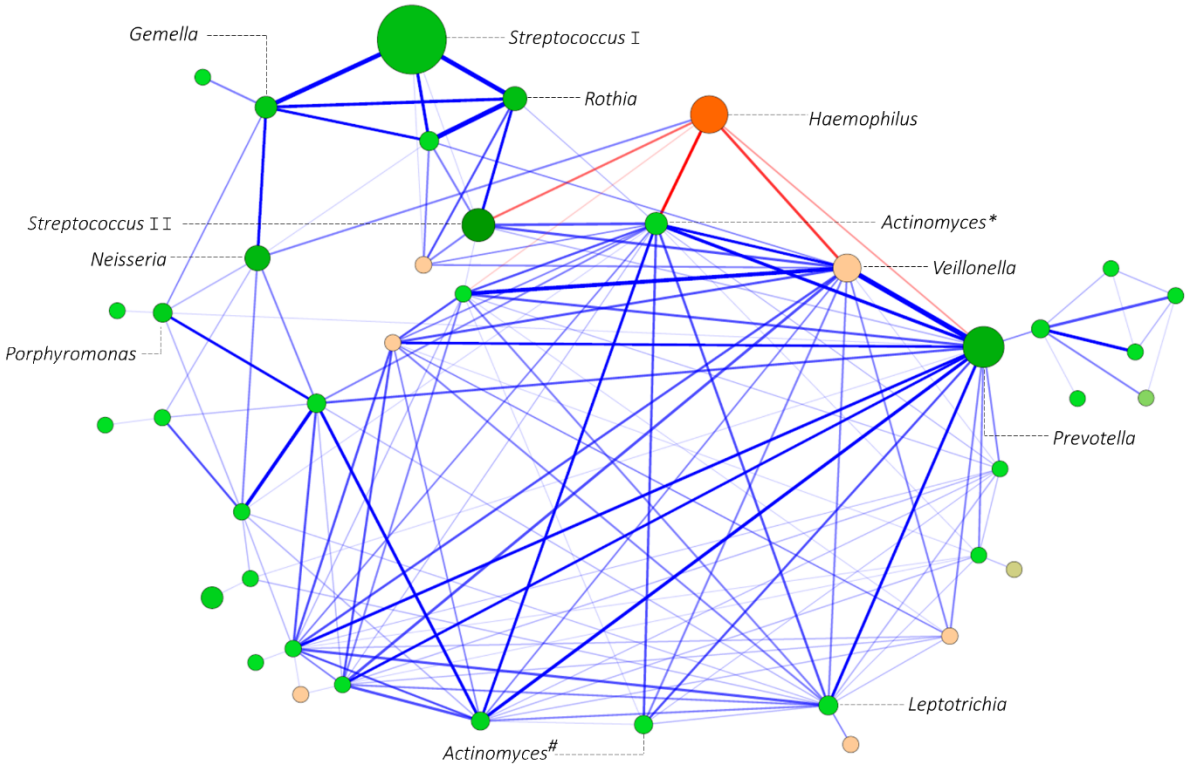

Figure 2.4: Bacterial network analysis of asthma cohort. Each edge represents a significant correlation coloured by either positive (blue) or negative (red). Edge width and transparency are proportional to the absolute value of the correlation coefficient. Node size is proportional to mean relative abundance. Node hue is proportional to the difference in taxon relative abundance between the neutrophilic phenotype group and the eosinophilic phenotype group. Correlations performed by SparCC with a correlation cut-off of R>0.25 or <-0.25. **Actinomyces* sp. uncultured bacteria, *#Actinomyces* sp. oral clone DR002.

Of the 13 discriminant taxa identified by SIMPER, *Streptococcus* II, *Gemella, Rothia* and *Porphyromonas* were significantly less abundant in neutrophilic than in eosinophilic and paucigranulocytic phenotypes (Figure 2.5A). Sputum neutrophil % positively correlated with the relative abundance of *Moraxella* and negatively correlated with the relative abundance of *Streptococcus* I, *Gemella* and *Porphyromonas* (Figure 2.5B). In contrast, *Haemophilus* negatively correlated with eosinophil %, and *Streptococcus* I, *Neisseria* and *Gemella* positively correlated with eosinophil % (see Figure E1.7 of Appendix 1). *Prevotella, Actinomyces, Leptotrichia, and Veillonella,* while identified by SIMPER and represented highly connected

nodes in the network analysis, did not differ between airway inflammatory phenotypes, nor correlate with sputum cell counts.

2.4.5 Non-dominant microbiome

The non-dominant microbiome was assessed to establish whether differences in microbiota composition between inflammatory phenotypes were explained solely by overgrowth of opportunistic taxa (e.g. *Haemophilus* and *Moraxella*) or, alternatively, whether differences existed even in the absence of pathogen predominance. Two separate approaches were used to investigate this; re-scaling of relative abundance data, following the exclusion of pathogen predominance, and assessment of ranked taxon fold change between neutrophilic and eosinophilic groups based on non-subsampled taxa counts. Re-scaled relative abundance data remained significantly different between inflammatory phenotypes ($p=0.0004$, pseudo-$F=2.38$). Pairwise tests revealed significant differences between neutrophilic and eosinophilic phenotype participants ($p=0.0001$, $T=2.31$, Table E1.5 of Appendix 1) and between neutrophilic and paucigranulocytic participants ($p=0.0002$, $T=2.26$, Table E1.5 of Appendix 1). Assessment of taxa count supported these findings, with significant differences in the fold change of taxa count between participants with neutrophilic and eosinophilic phenotypes (see Figure E1.8 of Appendix 1).

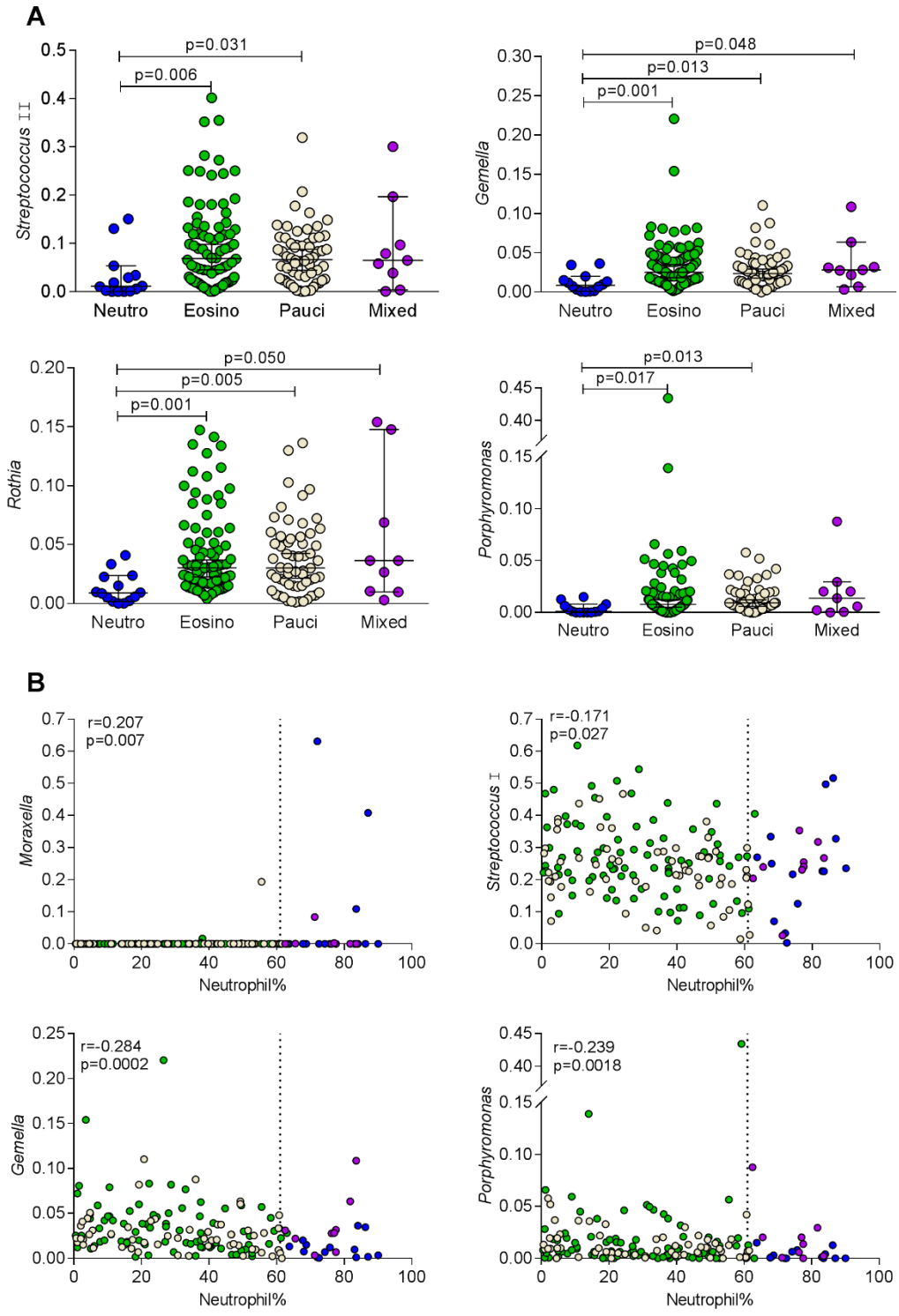

Figure 2.5: Taxa distribution differs by sputum neutrophilia. A) Taxa which significantly differ by patient inflammatory phenotype. B) Significant correlations between taxa and neutrophil %. Colours represent asthma phenotype, based on neutrophilia or eosinophilia. Dotted line at 61% neutrophils indicates phenotype cut-off point. Statistical significance was assessed by A) Kruskal-Wallis one-way ANOVA with Dunn's post hoc test and B) Spearman's rank correlation.

2.4.6 Clinical and inflammatory associations with microbiota composition

In univariate analysis, Faith's phylogenetic diversity significantly, inversely correlated with sputum neutrophil %, age, and ICS dose, and significantly, positively with FEV_1 % (Table 2.2). Conversely, weighted UniFrac distance from centroid significantly correlated, positively with sputum neutrophil %, and was significantly different based on gender and atopy but not with age, ICS dose, FEV_1 % or previous smoking status (Table 2.2). In multivariate analysis sputum neutrophil % was the only variable that independently predicted both Faith's phylogenetic diversity and weighted UniFrac distance from centroid (p=0.002 (95% CI=-0.07 to -0.02) and p<0.001 (0.07 to 0.22), respectively, Table 2.3). Age and ICS dose both independently predicted Faith's diversity (p=0.030 (-0.07 to -0.004) and p=0.042 (-0.001 to -0.001), respectively) while atopy and gender independently predicted distance from centroid (p=0.018 (1.3 to 8.4) and p=0.039 (-7.5 to -0.30), respectively).

Table 2.2: Comparison of patient characteristics with microbiota diversity

		Neutrophil %	Age	ICS Dose*	FEV_1 % predicted	Atopy†#	Gender†	Ever smoked†
Faith's diversity	r	-0.374	-0.309	-0.242	0.193			
	p	<0.001	<0.001	0.002	0.013	0.32	0.97	0.53
UniFrac Distance	r	0.24	0.015	0.096	0.034			
	p	0.002	0.84	0.22	0.66	0.019	0.003	0.70

Spearman correlation coefficient (r) and probability values are as indicated. *n=163, #n=164, †Assessed using Mann-Whitney test

Table 2.3: Multivariate linear regression between patient characteristics and microbiota diversity

		B	95% CI	p
Faith's phylogenetic diversity	Neutrophil %	-0.046	-0.07, -0.02	0.002
	Age	-0.036	-0.07, -0.004	0.030
	Gender	0.35	-0.59, 1.4	0.49
	Atopy	-0.77	-1.7, 0.12	0.10
	FEV_1 % predicted	0.016	-0.01, 0.04	0.23
	ICS dose	-0.001	-0.001, -0.001	0.042
UniFrac distance from centroid	Neutrophil %	0.14	0.07, 0.22	<0.001
	Age	0.056	-0.05, 0.16	0.30
	Gender	-3.9	-7.5, -0.30	0.039
	Atopy	4.8	1.3, 8.4	0.018
	FEV_1 % predicted	0.049	-0.04, 0.14	0.27
	ICS dose	<0.001	-0.001, 0.001	0.56

2.5 Discussion

To date, this is the largest study to date to assess predictors of the airway microbiota composition in asthma. The primary comparisons were between asthma inflammatory phenotypes, where significant differences were observed in the composition of airway microbiota. These differences were largely between neutrophilic and eosinophilic participants and reflected a reduced diversity and evenness of detectable bacterial taxa in the neutrophilic participants. Reduced microbiota diversity has been reported following acute and chronic airway infections in asthma (Green et al., 2014; Simpson et al., 2016b), and in other respiratory disorders (Cuthbertson et al., 2016; Hofstra et al., 2015; Pettigrew et al., 2016), as well as with the effects of exposure to antibiotics (Daniels et al., 2013; Huang et al., 2014). Importantly, none of the study participants reported clinical features of respiratory infection or had antibiotic therapy during the preceding month.

Alpha diversity metrics were further assessed relative to continuous neutrophil and eosinophil count data as an alternative to categorical inflammatory phenotypes. Significant correlations were observed between sputum neutrophil % and each assessed alpha diversity

metric, with no significant interactions between any diversity metric and sputum eosinophil %, strongly suggesting that decreased microbiota richness, evenness, and diversity are associated with airway neutrophilia. Analysis of sputum microbiota beta diversity (inter-sample similarity) also demonstrated substantial differences between people with neutrophilic airway inflammation and those with other inflammatory phenotypes, consistent with the stochastic overgrowth of complex commensal communities by individual opportunistic pathogens (Tarabichi et al., 2015).

Bacterial taxa that contributed to observed differences in microbiota composition between inflammatory phenotypes were identified, namely high abundance of *Haemophilus* and *Moraxella* in neutrophilic participants, supporting previous findings (Green et al., 2014). This could be interpreted as simply an increased relative abundance of airway pathogens in neutrophilic patients, reflecting neutrophilic influx into the airways during sub-clinical lower airway infection, with a reciprocal decrease in the relative abundance of commensal taxa. However, a group of common airway taxa correlated negatively with sputum neutrophil % (*Gemella*, *Porphyromonas*, and *Streptococcus*) and, importantly, even after high relative abundance of *Haemophilus* and *Moraxella* were accounted for, significant differences in the microbiota composition between neutrophilic and eosinophilic participants were still observed. This finding suggests that two separate phenomena could contribute to microbial differences between inflammatory phenotypes; the impact of pathogen overgrowth, and the selective pressure of airway inflammatory characteristics. With the former, pathogen overgrowth can impact the surrounding microbes through microbe-microbe interactions, independent of inflammatory phenotype. With the latter, a more broad-scale divergence in composition between neutrophilic and eosinophilic subgroups could, in turn, contributie to an increased risk of lower airway infection in neutrophilic patients through an increased presence of opportunistic pathogens.

This finding has clear implications for the clinical management of asthma, where low-dose macrolide and ICS therapies have been shown to influence overgrowth by opportunistic respiratory pathogens and innate immune function, respectively (Denner et al., 2016; Essilfie et al., 2012; Rogers et al., 2014). Further, the relative lack of efficacy of ICS in patients with non-eosinophilic asthma (Pavord et al., 1999) may lead to use of higher doses compared to eosinophilic patients. The combination of underlying differences in airway microbiota (associated with differences in inflammatory phenotype) and inefficacious therapies being used at higher doses might contribute to reduced bacterial diversity (Denner et al., 2016), the high concentrations of Proteobacteria seen in the airways of neutrophilic patients (Simpson et al., 2016b), a greater propensity for lung infection, and a further enhancement of the neutrophilic phenotype (Essilfie et al., 2012).

While strong associations between neutrophilic phenotype and sputum microbiota composition were found, associations between eosinophil counts and microbiota composition were minimal. This contrasts with previous studies reporting increased *Tropheryma* associated with eosinophilia (Simpson et al., 2016b), and associations between bronchial biopsy eosinophil count and bacterial composition (Huang et al., 2015), and reduced bacterial burden associated with type 2-high airway inflammation (Durack et al., 2016).

An important strength of this study was its involvement of a large group of well-defined participants with poorly controlled asthma, who were taking regular inhaled therapy. The application of detailed induced sputum microbiota characterisation from these participants then allowed us to assess the extent to which clinical and inflammatory characteristics independently associated with variations in airway microbiology using multivariate linear regression analysis. Multivariate regression identified sputum neutrophil % as the strongest predictor of microbiota variance. However, age, ICS dose, gender and atopy were also significant, independent predictors. Conversely, lung function (as measured by FEV_1 %) and

smoking status were not. Of particular interest was the finding that increasing age predicted reduced microbiota alpha diversity, as age has been previously associated with microbiome composition in other chronic respiratory diseases. The airway microbiome of CF patients is strongly affected by age (Zhao et al., 2012), which is presumed to relate to the selective effects of increased antibiotic exposure over time and the changing characteristics of the airway environment (Rogers et al., 2013). It is interesting to speculate that the relationship between neutrophilia and microbiota composition may reflect the effect of age on neutrophilia (Brooks et al., 2013), suggesting that a tendency towards a neutrophilic phenotype and/or a susceptibility to opportunistic airway infections increases with age in patients with severe asthma.

It is important to recognise a number of limitations of this study. Airway microbiology was assessed based on induced sputum, which, while shown to provide reproducible inflammatory cell levels in patients with moderate to severe asthma (Bacci et al., 2002; Rossall et al., 2014), only provides an approximation of lower airway microbiology (in common with other lower airway sampling strategies) (Rogers et al., 2010). It is also important to note that induced sputum levels of neutrophils and eosinophils may change frequently (Hancox et al., 2012; Suarez-Cuartin et al., 2016), and that the relationships between airway microbiology and inflammatory phenotype reported are cross-sectional. This study is a cross-sectional design and hence can only report association between the microbiome and airway inflammatory characteristics. Detailed longitudinal analysis is now required to determine how these relationships change with time. Inferences about causation may be able to be made in longitudinal and mechanistic studies, including more sophisticated metagenomic and functional studies, as well as intervention trials that attempt to manipulate the pulmonary microbiome. Further, 16S rRNA gene amplicon sequence data was subsampled to a level that allowed the inclusion of the greatest number of subjects, while maintaining sufficient read

depth to accurately describe microbiota composition. Additional analysis, using a greater read depth, might however identify rare taxa that contribute to disease characteristics. While multivariate regression identified sputum neutrophilia as an independent predictor of microbiota composition, the effects of variation in ICS dose between phenotypes (although non-significant) should be noted. Finally, though none of the study participants received antibiotics in the month prior to recruitment, data on less recent exposure were not available and could have a lasting impact on the lower airway microbiome composition (Daniels et al., 2013).

The clear relationship between airway inflammatory phenotype and microbiota highlight the need for studies examining whether asthma treatments should be individualised based on both inflammatory phenotype stratification and lower airway microbiology. There is now a clear need to investigate the extent to which variations in the airway microbiota predict the risk of future asthma exacerbations, and to determine whether airway microbiota characterisation could be used as a basis for asthma treatment selection.

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CHAPTER 3: *FUT2* GENOTYPE IN BRONCHIECTASIS

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>
> The supplementary information has been included in Appendix 2

3.1 Abstract

FUT2 encodes a protein that mediates attachment of α(1,2)-fucose to mucosal glycans. Common polymorphisms in the *FUT2* (secretor) gene have been found to influence infection susceptibility. Airway infections are a significant determinant of disease progression in patients with bronchiectasis. **I aimed to assess whether disease severity and airway microbiota composition differed according to *FUT2* genotype in bronchiectasis.**

The *FUT2* gene was sequenced for polymorphisms from 112 adult bronchiectasis patients from the BLESS trial. Loss-of-function phenotype was verified by histochemical staining in endobronchial biopsies. Disease parameters and baseline sputum bacterial, fungal, and viral components of the microbiota (measured by 16S rRNA gene amplicon sequencing, quantitative PCR, and quantitative reverse transcription-PCR respectively) were compared according to *FUT2* genotype.

Patients were grouped by *FUT2* loss-of-function genotype; categorised as non-secretors (n=27, *sese*), heterozygous secretors (n=54, *Sese*), or homozygous secretors (n=31, *SeSe*). FEV_1 % was significantly lower in *SeSe* patients compared to *sese* patients (mean=61.6 (SD=20.0) vs 74.5 (18.0); p=0.023). Exacerbation frequency was significantly higher in *SeSe* (mean count 5.77) compared to *sese* (4.07; p=0.004) and *Sese* (4.63; p=0.026) genotypes. The time until first exacerbation was significantly shorter in *SeSe* compared to *Sese* (HR=0.571

(95% CI=0.343-0.950); p=0.031), with a similar trend for *sese* patients (HR=0.577 (0.311-1.07); p=0.081). *sese* had a significantly reduced frequency of *Pseudomonas aeruginosa*-dominated airway infection (8.7%) compared to *Sese* (31%; p=0.042) and *SeSe* (36%; p=0.035). In contrast, fungal, viral, and non-dominant bacterial components of the microbiome were not significantly different between *FUT2* genotypes.

FUT2 genotype in bronchiectasis patients was significantly associated with disease outcomes, with homozygous secretors exhibiting lower lung function, higher exacerbation number, and a higher frequency of *P. aeruginosa*-dominated infection.

3.2 Introduction

Bronchiectasis is a chronic airway disease characterised by irreversible bronchial dilation and persistent bacterial infections (Cole, 1986). Owing to its multifactorial aetiology, a patient's individual disease progression is difficult to predict (Shoemark et al., 2007). However, the composition of the airway microbiota, which differs substantially between patients, correlates with clinical markers of disease severity (King et al., 2007; Rogers et al., 2013; Tunney et al., 2013).

Most notably, patients where *Pseudomonas aeruginosa* numerically dominates the composition of the airway microbiota present with an accelerated decline in lung function, more frequent pulmonary exacerbations, greater sputum production, and a greater requirement for antibiotic therapy (Evans et al., 1996; Ho et al., 1998; Rogers et al., 2014; Shoemark et al., 2007). Respiratory viral (Gao et al., 2015) and fungal (Maiz et al., 2015) infections have also been linked to bronchiectasis disease markers, which suggests that multiple infectious agents can contribute to the pathophysiology of bronchiectasis. While genetic loci in genes related to inflammation and airway remodelling have been previously investigated in bronchiectasis (Boyton et al., 2006; Chalmers et al., 2013; Daheshia et al., 2012; Hsieh et al., 2013; Stankovic

et al., 2009), little is known regarding common genetic polymorphisms that affect microbial acquisition in patients.

Variability in carbohydrate expression on mucosal surfaces is high, with some commensal and pathogenic microbes able to utilize glycans for adherence, induction of pathogenicity genes, and for use as carbon sources (Audfray et al., 2013; Marcobal et al., 2013; McGuckin et al., 2011). Therefore variability in glycan expression in the respiratory tract affects susceptibility to infection with many bacteria, viruses, and fungi (Rose and Voynow, 2006). The *FUT2* (*secretor*) gene encodes an α(1,2)-fucosyltransferase, and homozygous loss-of-function mutations result in the inability to express ABH, Lewis^{b}, and Lewis^{y} glycans on mucosal surfaces (Ferrer-Admetlla et al., 2009). Individuals who carry at least one functional copy of the *FUT2* gene are known as "secretors" (*Sese* or *SeSe* based on one or two functional copies, respectively) while those with two non-functional copies (approximately 20% of the Caucasian population) are known as "non-secretors" (*sese*) (Ferrer-Admetlla et al., 2009; Genomes Project et al., 2012).

Secretion of α(1,2)-fucosylated glycans elicits a dichotomous effect on host-microbe interactions, the result of which is a difference in infection susceptibility, disease susceptibility, and microbiome composition (Rausch et al., 2011) between secretors and non-secretors. For example, non-secretors have reduced incidence of influenza A, influenza B, rhinovirus, and respiratory syncytial virus infections (Raza et al., 1991), but increased incidence of *Neisseria meningitidis*, *Streptococcus pneumoniae* (Blackwell et al., 1986), and *C. albicans* (Thom et al., 1989) infections, compared to secretors. In relation to chronic respiratory conditions, non-secretor asthma patients present with fewer exacerbations (Innes et al., 2011) and non-secretor cystic fibrosis patients (with severe impairment of lung function) have prolonged time until *P. aeruginosa* colonization (Taylor-Cousar et al., 2009); however non-secretors with COPD have a lower FEV_1 % (Cohen et al., 1980). While one functional copy of *FUT2* is sufficient to

facilitate α(1,2)-fucosylation, it is currently unclear whether heterozygote secretors (*Sese*) exhibit the same infection and disease susceptibility patterns as homozygote secretors (*SeSe*), or whether incomplete dominance presents, whereby heterozygotes display an intermediate phenotype.

I hypothesised that secretor genotype is a factor underlying variation in infection type and disease severity in bronchiectasis. To address this, I determined secretor genotype in a randomised controlled trial cohort of 112 adult bronchiectasis patients and performed secondary analysis by determining associations between genotype and key measures of disease severity. Additionally, I assessed whether secretor genotype was associated with changes in bacterial, viral and fungal components of the airway microbiota.

3.3 Methods

3.3.1 Study population

Bronchiectasis patients were recruited as part of The Bronchiectasis and Low-dose Erythromycin Study (BLESS) randomised controlled trial (Serisier et al., 2013). This trial assessed the effect of 12 months of low dose erythromycin therapy (twice-daily erythromycin ethylsuccinate; 400mg) on exacerbation rates in adults with bronchiectasis. Adult patients aged 20–85 years with high-resolution CT scan-proven bronchiectasis, two or more exacerbations in the previous 12 months and daily sputum production were eligible. Patients were not receiving systemic corticosteroids, had not smoked cigarettes in the preceding six months, were macrolide-naïve, and were not receiving nebulized antibiotics. Full details of inclusion and exclusion criteria for the study are detailed in the supplementary methods of Appendix 2.

3.3.2 Clinical sample collection and processing

Sputum induction was performed as follows: Subjects were instructed to perform their usual chest physiotherapy regime on the morning of the sputum induction procedure. Prior to commencement of hypertonic saline inhalation, any spontaneous sputum expectorated was collected for standard culture. Sputum induction was performed after inhalation of 400 ug of albuterol, using 4.5% hypertonic saline nebulised from an ultrasonic nebuliser (output >1 mL/ min) for 20 minutes in 4 periods of 5 minutes each, according to the standardised protocol recommended by the European Respiratory Society taskforce (Paggiaro et al., 2002). Following mouth-rinsing and expectoration, sputum was collected following each nebulisation period, on each occasion preceded immediately by spirometry. The first sputum sample was refrigerated immediately following collection and frozen at -80 °C within an hour. A cold chain was maintained up until the point of DNA extraction.

Ten, 15 and 20-minute samples were pooled and an aliquot from this placed on ice immediately and transferred for inflammatory cell count processing within 60 minutes. Sputum was processed according to the methods of the US Cystic Fibrosis Therapeutics Development Network Standard Operating Procedure (Sagel et al., 2001). Briefly, an equal volume of sterile 10% dithiothreitol (Sputolysin; Calbiochem-Novabiochem Corp., San Diego, CA), was added to the sputum, then incubated in a shaking water bath at 37° C for 5-10 min, and mixed using a transfer pipette at 5-min intervals. A further three times the volume of both dithiothreitol and phosphate-buffered saline (Dulbecco's; Gibco BRL, Grand Island, NY) was added and the mixture incubated again in the 37° C shaking water bath for another 5-10 min. 10 µl of homogenized sputum samples, mixed with Trypan Blue, was used to calculate total cell counts using a standard hemacytometer. A further 0.25-0.50 ml of both samples was used to prepare cytospin slides for differential cell counts. After staining the slides with Wright's stain, 300 non-squamous cells were counted and cell differentials calculated.

Endobronchial Biopsy Collection and Processing was performed as follows: Subjects were fully informed about the potential risks of the procedure and provided written consent. Bronchoscopy was performed as an outpatient procedure in the endoscopy unit of the operating theatres of the Mater Adult Hospital, South Brisbane, Australia using an Olympus flexible fibre-optic bronchoscope according to the safety standards of the Thoracic Society of Australia and New Zealand (Wood-Baker et al., 2001), with details of the research bronchoscopy procedure adapted from prior methods (Hattotuwa et al., 2002; Hilliard et al., 2002; Monton et al., 1999). Subjects fasted for 6 hours before the procedure. The procedure was performed transorally, under light sedation using intravenous midazolam and fentanyl to ensure patient comfort. Topical lignocaine was applied to the vocal cords and bronchi by instillation through the bronchoscope. Endbronchial biopsies were then taken from subsegmental carinae of the lower lobes using Boston scientific Radial Jaw 3 single-use biopsy forceps (diameter 1.8 mm), starting at 5th order airways and working proximally as far as the 3rd order bronchi if necessary (bifurcation of segmental and subsegmental bronchi). Subjects were observed for 2 hours after the bronchoscopy before being allowed home.

3.3.3 Clinical measures of respiratory function and disease severity

Clinical assessments included forced expiratory volume in one second, as a percentage of predicted value (FEV_1 %) (O'Donnell et al., 1998), Leicester Cough Questionnaire (LCQ) score (Birring et al., 2003), St. George's Respiratory Questionnaire (SGRQ) score (Jones et al., 1992), sputum weight over a period of 24 hours, physician defined pulmonary exacerbations over the 48 weeks of the trial (PDPEs), and pulmonary exacerbations in the prior 12 months (treated with either oral or intravenous antibiotics). During exacerbations, oral antibiotics were typically prescribed for milder exacerbations, where the patients were deemed well enough to return home, while intravenous antibiotics were prescribed if patients were deemed by the treating physician to have a severe exacerbation.

All clinical measures analysed in this study were obtained prior to commencement of the clinical trial, except PDPEs which were recorded over the 48 weeks of the trial. Data relating to PDPEs were therefore presented for the total cohort and for those receiving placebo alone as a sensitivity analysis, in order to remove the possibility of confounding from macrolide use during the trial.

3.3.4 FUT2 Polymorphism Genotyping

Genomic DNA was extracted from patients' serum using Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich), according to manufacturer's instructions. An 1162 bp region of the *FUT2* gene was amplified using primers F: 5'-CGTGTCCCGTTTTCCTCCCC, R: 5'-AGAGAGATGGGTCCTGCTCAT. Each reaction contained 5 μL of KAPA Taq HotStart Buffer (KAPA Biosystems, MA, USA), 2.5 μL of MgCL2, 0.5 μL of dNTP, 0.5 μL of each primer (10 μM), 0.2 μL KAPA Taq HotStart polymerase, 14.8 μL dH2O and 1 μL of genomic DNA. Cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 30s, 60°C for 15s, 72°C for 30s. A final elongation was allowed at 72°C for 5 min. Successful amplification was confirmed by gel electrophoresis, and the PCR products were purified using a DNA acid phosphatase-exonuclease kit (New England BioLabs, MA, USA) according to the manufacturer's instructions.

Amplicons were then sequenced by Sanger Sequencing using two internal primers (5'-TGCTGGTCGTTCAGATGCCT and 5'- CCATCTTCAGAATCACCCTG) by Flinders Sequencing Facility (SA Pathology, Bedford Park, South Australia). Readouts were aligned to a reference sequence using Clustal W multiple alignment using UGENE, where polymorphisms were detected by mismatched alignment. Polymorphisms were then cross-referenced with the literature to determine loss-of-function polymorphisms.

3.3.5 Secretor Status Bronchial Phenotyping by Immunohistochemistry

Horseradish peroxidase conjugated *Ulex Europaeus lectin 1* (HRP-UEA1) (EY Laboratories, San Mateo, CA, USA) staining was performed on 32 endobronchial biopsies. After removal of paraffin and rehydration of biopsies, endogenous peroxidase was inhibited by 20 min incubation in 3.0% H2O2. Sections were rinsed twice for 5 min in phosphate buffered saline (PBS)/0.3% Tween-20. Tissue was blocked with 200 µl of 1% bovine serum albumin/PBS for 30 min at room temperature. Sections were then incubated with 200 µl of 5 µg/mL HRP-UEA1 for 1 hour. Sections were then rinsed in PBS/Tween-20, 3 times for 5 min, and AEC substrate (Vector Laboratories, Burlingame, CA, USA) added for 30 min before addition of haematoxylin counterstain (all steps at room temperature).

3.3.6 Bacterial dominance

Sputum DNA extraction and 16S rRNA gene sequencing were performed in 93 of the 112 patients, based on the availability of suitably archived samples, as described in Chapter 2.3 using a combined physical, enzymatic, and heat-based cell lysis, followed by phenol-chloroform extraction and DNA recovery using EZ-10 Spin columns (Bio Basic, Inc., Ontario).

Identification of numerically dominant species (using specific PCR-based assays) were performed prior to this study and described previously (Rogers et al., 2014). Patients were also categorised into one of three groups previously, based on the numerically dominant bacterial species (Rogers et al., 2014). The groups comprise of *P. aeruginosa*-dominated, *Haemophilus influenzae*-dominated, or dominated by a species other than these two. Previous quantification of the relative abundance of bacterial genera in this patient cohort has revealed that the airway microbiota is highly polarised in patients with *H. influenzae*– and *P. aeruginosa*–dominated infections, with dominance of either exceeding 80% abundance (Rogers et al., 2013). A summary of the dominant microbes in the patient cohort is provided in Table E2.1 of Appendix

2. For analysis of the non-dominant microbiota, *Pseudomonas* or *Haemophilus* taxa was removed (where dominant) and the remaining relative abundance measures rescaled, as described previously (Rogers et al., 2015). Sequence reads have been deposited to the Sequence Read Archive database, hosted by the National Center for Biotechnology Information, under the study accession number SRA066194.

3.3.7 Fungal detection

Detection of *C. albicans* and *A. fumigatus* were performed by quantitative PCR in 78 patient samples, where suitably archived samples were available. Oligonucleotides and thermocycling conditions were performed as previously described.(Innings et al., 2007; Rantakokko-Jalava et al., 2003) Detection limits were defined by serial dilutions of the clinical isolates and approximate copy numbers calculated (see Table E2.2 of Appendix 2).

3.3.8 Viral detection

Detection of nine common respiratory viruses (rhinovirus, adenovirus, influenza A, influenza B, parainfluenza 1, 2, 3, respiratory syncytial virus, and human metapneumovirus) was assessed in baseline induced sputum from the same 78 patients as those where fungal detection was performed. Detection was performed by the diagnostic virology unit, SA Pathology, using routine clinical diagnostic quantitative reverse-transcriptase PCR assays. Sample extraction was performed on a MagNA Pure© 96 automated workstation (Roche Diagnostics, Castle Hill, NSW) and amplification and detection using specific in house primers and probes in validated PCR assays was performed on a LightCycler© 480 with an extraction control and multiplexed assays with a maximum of three targets per reaction.

3.3.9 Statistical analysis

Clinical patient characteristics were tested for non-normality including skewness and kurtosis using the D'Agostino & Pearson omnibus test. Continuous data were analysed by one-

way analysis of variance (ANOVA) with Tukey's post-hoc test, or by Kruskal-Wallis with Dunn's post-hoc test, according to the distribution of the data. Ordinal data were analysed by Wilcoxon rank-sum test. Exacerbation counts were tested for Poisson distribution and subsequently analysed using a Wald test. Categorical data were analysed by Chi-square or Fisher's exact test as appropriate. For time until first PDPE, Kaplan-Meier curves were produced, and Cox proportional-hazards regression analyses were performed. All analyses were performed using SPSS (version 23.0, IBM, Armonk, NY) or GraphPad Prism (version 6.05; GraphPad Software, San Diego, CA).

Alpha diversity (Simpson's Index, Shannon-Weiner Index) and beta diversity (Bray-Curtis similarity matrices) measures were calculated using sample-normalised, square root transformed relative operational taxonomic unit (OTU) abundance using PRIMER (version 6.1.16; PRIMER-E Ltd, Plymouth, UK). Principal coordinate (PCO) analysis was used to visualize clustering of samples based on their similarity matrices. The two-factor permutational multivariate analysis of variance (PERMANOVA) on the Bray-Curtis matrix was performed using PERMANOVA+ package for PRIMER with 9,999 random permutations.

3.4 Results

3.4.1 Secretor genotype and phenotype

FUT2 exon 2 genotype was determined in 112 patients, of whom 27 (24%) had a homozygous rs601338 428G→A base change (non-secretors), 54 (48%) were heterozygotes, and 31 (28%) were homozygous for no mutation, termed *sese*, *Sese*, and *SeSe* respectively. The minor allelic frequency of this was 0.48 and the proportions fit the Hardy-Weinberg distribution. Secretor phenotype, determined by histochemical staining of bronchial tissue, aligned with the genotype in all 32 patients with available biopsies (see Figure E2.1 of Appendix 2).

3.4.2 Clinical characteristics

Age, gender, duration of disease, drug treatments, and comorbidities did not differ significantly between secretor genotypes (Table 3.1). However, of the clinical measures of disease severity, homozygous secretor patients (*SeSe*) had significantly lower FEV_1 (% predicted) compared to non-secretor patients (*sese*) (mean 61.6 (95% CI=54.3 to 69.0) vs 74.5 (67.3 to 81.6), p=0.023, Table 3.1, Figure E2.2 of Appendix 2).

Table 3.1: Patient demographic and clinical characteristics

	sese (n=27)	Sese (n=54)	SeSe (n=31)	p value
Age (years)	64.0 (58-68)	64.5 (59-71)	64.0 (60-67)	0.77
Females, n (%)	14 (52)	36 (67)	18 (58)	0.41
Duration of bronchiectasis (years)	50.0 (12-55)	50.0 (16-60)	55.0 (33-60)	0.43
Pre-bronchodilator FEV_1 (L)	2.05 (0.64)	1.74 (0.60)	1.67 (0.72)	0.060
Pre-bronchodilator FEV_1 (% predicted)	74.5 (18.0)	68.2 (17.0)	61.6 (20.0)	0.030
Post-bronchodilator FEV_1 (L)	2.18 (0.65)	1.83 (0.63)	1.75 (0.71)	0.035
Post-bronchodilator FEV_1 (% predicted)	79.1 (18.5)	71.6 (17.9)	64.5 (19.2)	0.013
24-hour sputum weight (g)	19.8 (10.3)	18.5 (12.1)	11.0 (13.8)	0.051
St George's Respiratory Questionnaire score	34.5 (13.7)	38.8 (15.0)	37.5 (15.3)	0.46
Leicester Cough Questionnaire score	14.3 (3.35)	15.0 (3.05)	15.3 (3.09)	0.43
6 min walk test (m)	500 (97.5)	519 (92.0)	513 (80.6)	0.67
C-reactive protein concentration (mg/L)	3.10 (0.75-6.8)	3.25 (1.1-8.7)	3.60 (1.6-8.4)	0.56
Serum immunoglobulin concentration (g/L)	11.8 (3.17)	10.6 (2.34)	12.4 (3.78)	0.092
Sputum neutrophils (% of non-squamous cells)	96.1 (87-98)	96.7 (94-98)	96.0 (93-97)	0.59
Days on Antibiotics (days)	10 (0-26)	15 (0-36)	21 (6-40)	0.250
Exacerbations				
In the year prior to trial	4.07 (2.11)	4.63 (2.68)	5.77 (3.85)	0.011
Required IV antibiotics in the year prior to trial	0.00 (0.00)	0.19 (0.44)	0.45 (0.82)	0.031
Physician defined pulmonary exacerbation during trial (total)	1.15 (1.17)	1.74 (2.05)	2.03 (1.45)	0.033
Physician defined pulmonary exacerbation during trial (placebo group)	1.30 (1.18)	2.30 (2.51)	2.21 (1.58)	0.11
Drug treatments, n (%)				
Combination (ICS plus LABA)	13 (48)	24 (44)	13 (42)	0.89
Inhaled LABA alone	1 (4)	2 (4)	1 (3)	0.99
Inhaled SABA alone	7 (26)	27 (50)	15 (48)	0.10
Inhaled anticholinergic drugs	2 (7)	7 (13)	5 (22)	0.60
Inhaled corticosteroids alone	2 (7)	6 (11)	5 (16)	0.58
Prednisolone	0 (0)	2 (4)	1 (3)	0.62
Nebulised saline	1 (4)	1 (2)	0 (0)	0.57
Inhaled mannitol	0 (0)	1 (2)	0 (0)	0.58
Comorbidities, n (%)				
Asthma	6 (23)	7 (14)	7 (23)	0.45
Ciliary dysfunction	1 (4)	1 (2)	1 (3)	0.87
Hypertension	8 (30)	21 (39)	8 (26)	0.43
Ischaemic heart disease	1 (4)	8 (15)	2 (6)	0.22
Cerebrovascular disease	0 (0)	5 (9)	1 (3)	0.18
Diabetes mellitus	0 (0)	1 (2)	2 (6)	0.28

Erythromycin treatment during trial, n (%)#	14 (52)	31 (57)	12 (39)	0.25

Data are mean±SD, n (%), or median (IQR). FEV1=forced expiratory volume in 1 second. FEV1, % predicted=FEV1 as a percentage of the predicted value. ICS = inhaled corticosteroid, LABA = long-acting β agonist, SABA = short-acting β agonist. P values calculated by one-way ANOVA, Kruskal-Wallis, Wald, or Chi-square test according to the characteristics of the data distribution. #Erythromycin treatment commenced during the trial period and only relates to total physician defined pulmonary exacerbations.

The number of exacerbations over the 12-month period prior to the trial intervention was also significantly higher in *SeSe* compared to *sese* (incident rate ratio (IRR)=1.42 (95% CI=1.11 to 1.78), p=0.004), and *Sese* (IRR=1.25 (1.03 to 1.51), p=0.026) patients (Figure 3.1A). Of those, exacerbations requiring IV antibiotics (broadly indicative of a more severe pulmonary exacerbation) also differed, with no *sese* patients recording an exacerbation which required IV antibiotic therapy over the 12 months prior to the trial compared to 29% of *SeSe* patients having at least one exacerbation requiring IV antibiotics (Figure 3.1B).

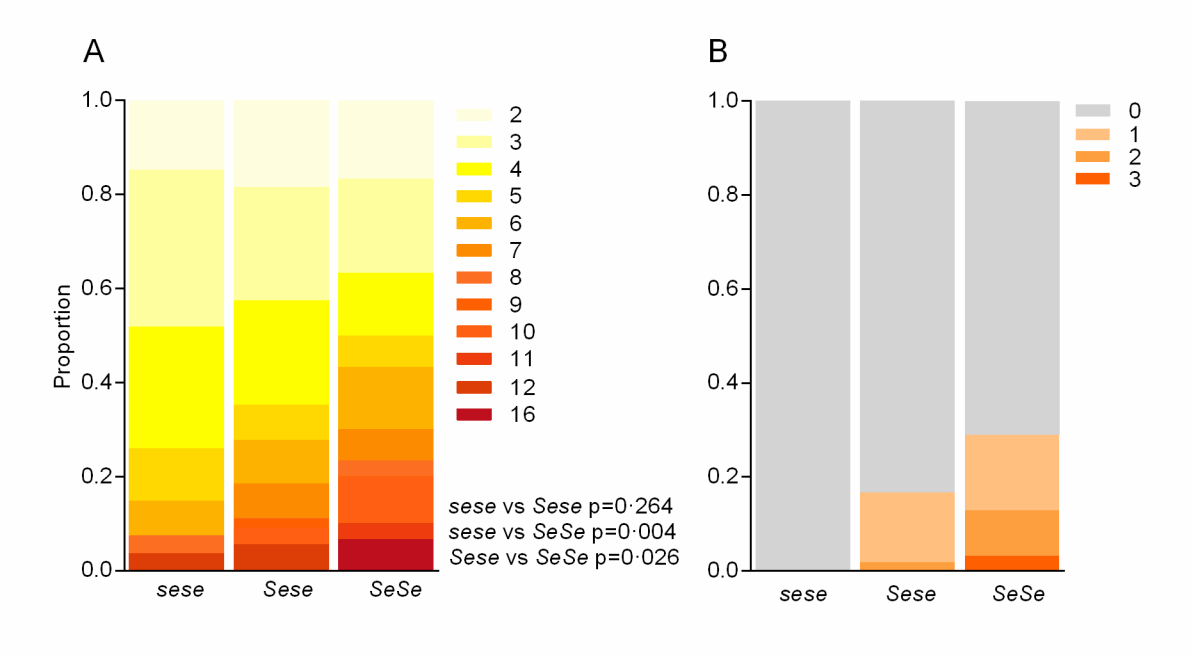

Figure 3.1: Effect of *FUT2* on 12-month pulmonary exacerbation count. A) Proportion of patients who had pulmonary exacerbations requiring any antibiotics and B) which required intravenous antibiotics over a 12-month period. Colour indicates number of exacerbations. P values calculated by Wald test.

Over the course of the clinical trial, the primary outcome measures were the number of PDPE, as well as the time until the first PDPE. Significantly fewer *sese* patients recorded PDPEs compared to both *Sese* (IRR=1.52 (1.01 to 2.28), p=0.045) and *SeSe* (IRR=1.77 (1.15 to 2.72), p=0.009) patients (Table 3.1). The time until the first PDPE was also significantly longer in the *Sese* compared to *SeSe* genotype (hazard ratio (HR)=0.571 (95% CI=0.343 to 0.950), p=0.031), with a similar trend for *sese* patients compared to *SeSe* (HR=0.577 (0.311 to 1.07), p=0.081) (Figure 3.2A). A similar pattern was also found in the placebo subgroup, however this did not reach statistical significance (*Sese* vs *SeSe*, HR=0.663 (0.329 to 1.34), p=0.252, *sese* vs *SeSe* HR=0.669 (0.305 to 1.60), p=0.396, Figure 3.2B). As many of the PDPEs were treated with antibiotic therapy, the total number of days on prescribed antibiotics was also tested, however was not significantly lower in *sese* patients compared to *Sese* (p=0.502) or *SeSe* (p=0.094) patients (Figure E2.3 and Figure E2.4 of Appendix 2).

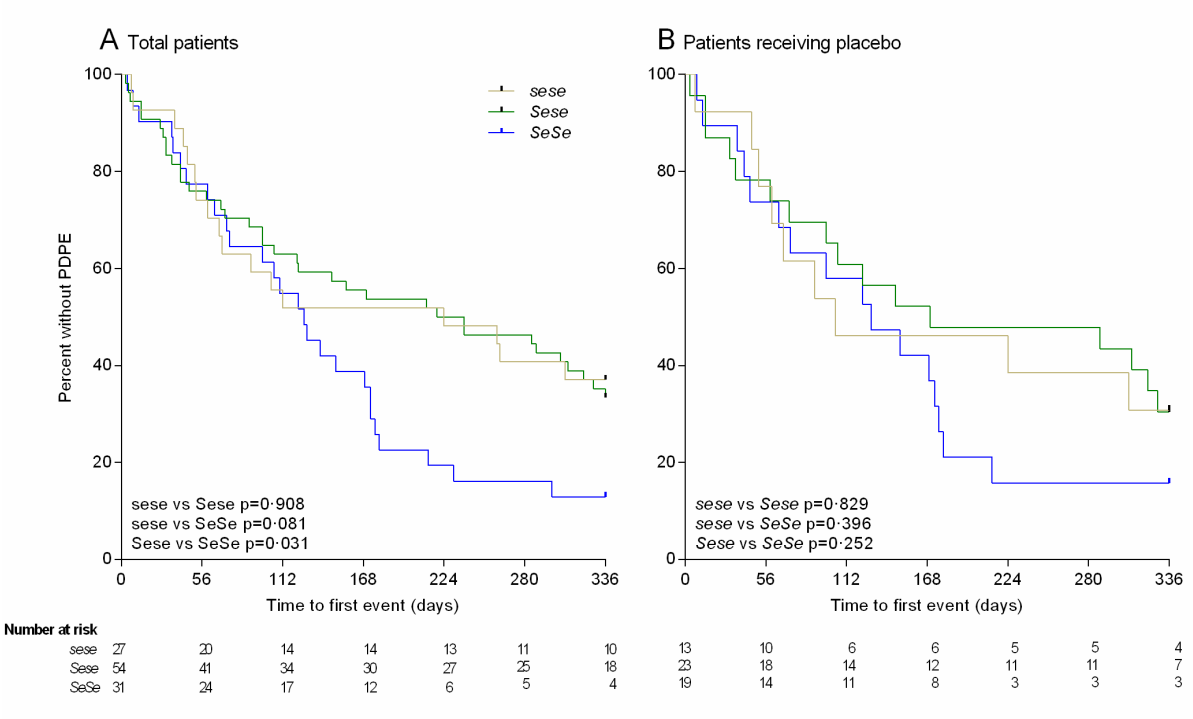

Figure 3.2: Effect of *FUT2* on time to first physician defined pulmonary exacerbation (PDPE). A) Total patients B) patients receiving only placebo. Kaplan-Meier curves are shown for the probability of remaining exacerbation-free according to *FUT2* genotype. p values calculated by Cox proportional-hazards regression model.

3.4.3 Airway bacterial predominance

Of the 93 patients where airway bacterial composition was determined (23 *sese*, 48 *Sese*, and 22 *SeSe*), 25 had infections dominated by *P. aeruginosa*, 33 by *H. influenzae* and 35 by any other species. The proportion of patients with *P. aeruginosa*-dominated infections was significantly lower in *sese* patients compared to *Sese* (p=0.042) and *SeSe* (p=0.035) patients (Figure 3.3).

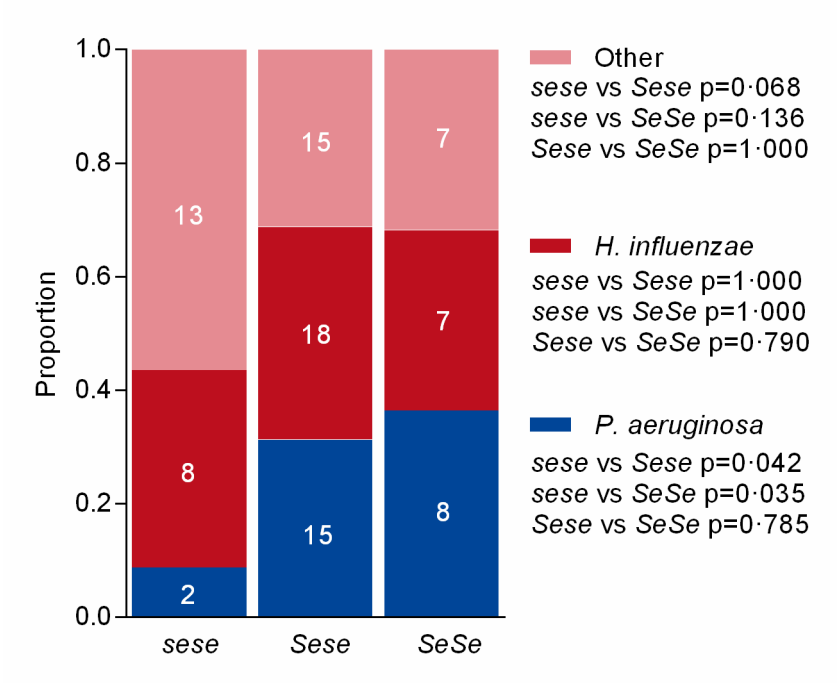

Figure 3.3: Effect of *FUT2* on predominant airway infection. Proportion of patients who had airway infection dominated by *Pseudomonas aeruginosa, Haemophilus influenzae*, or any other species. Numbers indicate number of patients. p values calculated by Fisher's exact test.

3.4.4 Airway non-dominant bacterial taxa

To determine whether non-dominant components of the microbiota differed according to secretor genotype, differences in the non-dominant bacterial taxa, key fungal pathogens, and key viral pathogens were compared. For non-dominant bacterial taxa, Shannon-Weiner Index and Simpson's Index were used to assess alpha diversity. Neither differed by secretor genotype (p=0.78 and p=0.73, respectively, see Figure E2.5 of Appendix 2). Principal Coordinate Analysis (PCoA) of Bray-Curtis distances revealed no distinct clustering between secretor genotypes, which was confirmed by PERMANOVA test on the Bray-Curtis dissimilarity matrices (p=0.78, see Figure E2.6 of Appendix 2), indicating that the composition of the non-dominant taxa did not significantly differ between patients based on *FUT2* genotype.

3.4.4 Airway fungal and viral infection

Of the 78 patients where *C. albicans* and *A. fumigatus* were quantified (17 *sese*, 39 *Sese*, and 22 *SeSe*), *C. albicans* was detected in 19/78 (24%) patients and *A. fumigatus* in 9/78 (11%) patients, although there was no difference in fungal presence between secretor genotypes (all $p>0.05$, see Figure E2.7 of Appendix 2). Similarly, of the nine viruses screened, only human rhinovirus was detected, in 4/78 (5%) patients, and there was no association with *FUT2* genotype ($\chi^2=1.5$, $p=0.48$).

3.5 Discussion

Investigations of secretor status have not been previously performed in bronchiectasis patients. In this cohort, the *FUT2* G428A single nucleotide polymorphism (rs601338) allelic frequency was at 0.48. The rs601338 frequency reported in the healthy Caucasian population is 0.44 (Ferrer-Admetlla et al., 2009; Genomes Project et al., 2012); similar to the frequency reported in this cohort and suggesting that secretor status does not affect development of non-cystic fibrosis bronchiectasis. However, within bronchiectasis, the results show that secretor genotype significantly affects disease severity.

By stratifying the bronchiectasis cohort by presence of *FUT2* null allele, it was revealed that non-secretor patients (*sese*) had significantly higher lung function and lower frequency of pulmonary exacerbations, compared to homozygous secretor (*SeSe*) patients. Differences in patients' dominant airway microbiology (but not non-dominant microbiology, presence of common fungal species, or detection of viral infections) was also found, with *sese* patients exhibiting significantly decreased frequency of *P. aeruginosa*-dominated airway infections.

Polymorphisms in the *FUT2* gene are conserved at a high frequency in the population, likely driven by a dichotomous effect of α(1,2)-fucosylated glycans on mucosal infection and

disease susceptibility. In asthma for example, the frequency of a non-secretor phenotype is higher among Caucasian asthmatics compared to non-asthmatics (Ronchetti et al., 2001), however within asthma patients, those with a secretor genotype are more prone to exacerbations (Innes et al., 2011), analogous to the findings of this study. In COPD, patients who are non-secretors have a lower FEV_1 % compared to secretors (Cohen et al., 1980). Finally, in cystic fibrosis, no difference in lung function has been found between secretor and non-secretor patients, however secretor patients in the smaller subset of "severe cystic fibrosis" patients have earlier onset of persistent *P. aeruginosa* infection compared to severe, non-secretor, cystic fibrosis patients (Taylor-Cousar et al., 2009), which is consistent with the findings of this study in bronchiectasis. It is also worth noting that along with chronic respiratory diseases, secretor status also significantly influences susceptibility to many gastrointestinal infections and diseases (Marcobal et al., 2013).

The results of this study are also indicative of an incomplete dominance genetic model for secretor status. Heterozygous patients displayed an intermediate phenotype in terms of lung function, total exacerbation frequency and frequency of exacerbations requiring IV antibiotics, despite *Sese* genotype considered equivalent to *SeSe* genotype. Previous findings also support incomplete dominance for *FUT2*, where similar, intermediate *Sese* genotype effects were found in relation to Crohn's disease gut microbiome and premature infant mortality (Morrow et al., 2011; Tong et al., 2014). Further research is required to determine the effect of heterozygous versus homozygous secretor status on glycan expression and infection/disease susceptibility.

The precise mechanism by which secretor status affects infection and disease susceptibility is not entirely clear. It has been previously shown that even healthy *sese* individuals have a distinct intestinal microbiota composition from healthy *Sese* or *SeSe* individuals (Rausch et al., 2011), linked to selective pressure from availability of different carbon sources (Kashyap et al., 2013). Secretor status could therefore affect infection and

disease susceptibility via its effects on microbiome composition, as the microbiome has been shown to influence host immunity. Mechanistic insight for several gastrointestinal pathogens (including *Helicobacter pylori* and Norwalk virus) have been characterised in which specific binding to α(1,2)-fucosylated glycans has been characterised (McGuckin et al., 2011).

This cohort showed an increased frequency of *P. aeruginosa*-dominated infection in both *Sese* and *SeSe* bronchiectasis patients. However, unlike *H. pylori* or Norwalk virus, *P. aeruginosa* does not encode any fucose catabolising genes and its fucose-specific adherence genes, PA-IIL and FliD, are not specific for α(1,2)-fucosylated glycans (Scharfman et al., 2001; Wu et al., 2006), suggesting no direct link between secretor status and *P. aeruginosa* infection. Further detailed characterisation of *P. aeruginosa* growth, adherence, and gene expression in sputum from individuals with *sese* and *Sese*/*SeSe* genotypes would be required to determine whether the effects of *FUT2* provide any selective pressure. Other taxa which make up the respiratory microbiota could also be influenced by the presence of secretor glycans but were too subtle to detect by relative abundance analyses.

Alternatively, positive secretor status may affect severity of bronchiectasis via the reported association with susceptibility to viral infections. Gastrointestinal viruses (Imbert-Marcille et al., 2014) and respiratory viruses such as influenza A, influenza B, rhinovirus, and respiratory syncytial virus (Raza et al., 1991) are all more prevalent in individuals with a functional *FUT2* gene. Respiratory viral infections are associated with pulmonary exacerbations in patients with bronchiectasis (Gao et al., 2015). Pulmonary exacerbations are characterised by inflammation, lung damage (which often permanently impairs lung function), and bacterial overgrowth, often requiring antibiotic therapy. A higher antibiotic burden has in turn been shown be selective for *P. aeruginosa* acquisition (King et al., 2007).

While further investigations are required to adequately test this, a model is proposed, whereby patients with a *Sese* or *SeSe* genotype have a greater susceptibility to respiratory viral infection, resulting in increased pulmonary exacerbation frequency, and leading to lower lung function, increased antibiotic exposure, and subsequent selection for *P. aeruginosa*. These results support this as secretors, particularly *SeSe* patients, had higher frequency of pulmonary exacerbations, lower FEV_1 %, more days on antibiotic therapy, and higher frequency of *P. aeruginosa* predominance, compared to *sese* patients. While results of the viral analysis showed a low prevalence of respiratory virus carriage across all groups, samples were taken from patients at a stable clinical baseline and are likely to differ in the period prior-to or during exacerbations. Longitudinal viral detection at baseline and during exacerbations, would be required to adequately address this hypothesis and signifies a potential mechanism contributing to divergences in disease progression among bronchiectasis patients.

Fungal colonisation was also assessed in this patient cohort. Oral and vaginal carriage of *Candida spp.* have been previously found to be higher in non-secretor individuals (Burford-Mason et al., 1988; Thom et al., 1989). Also, *A. fumigatus* encodes a lectin (AFL) which preferentially binds α(1,2)-fucosylated Lewis glycans (Houser et al., 2013), indicating secretor status may affect presence of both *C. albicans* and *A. fumigatus* in bronchiectasis sputum. However, neither presence of *C. albicans* nor *A. fumigatus* differed significantly based on secretor genotype, suggesting that fungal infections do not contribute to the difference in clinical symptoms reported between secretors and non-secretors.

Overall, the results of this study indicate that stratifying bronchiectasis patients based on secretor genotype is likely to provide substantial prognostic value. Secretor status, which can be determined rapidly and at low cost, could be an important determinant of frequency of respiratory events, such as pulmonary exacerbations. Such information is not only relevant for

clinical management, but also an important consideration in clinical trial design and the potential efficacy of vaccine strategies in different secretor groups.

These results are not without limitation. Since the original trial was first published, bronchiectasis severity scores have been developed that provide a cumulative marker for disease. These scores have been shown to be useful for defining bronchiectasis severity, however could not be calculated for this cohort. The main reason for this is the absence of a bronchiectasis score from radiological examination. While CT scans from the patients were performed to confirm presence of bronchiectasis, these were not stored or scored for severity and cannot be retrospectively determined. Another limitation is that the data presented is from a small number of patients based from a single centre study with no external validation cohort. Furthermore, the inclusion criteria for patients in the original study selected represent a severe population, limiting the ability to demonstrate differences in severity of disease between the secretor types. Repeating this study in different, independent bronchiectasis populations, including a wider range of severities and potentially different bronchiectasis aetiologies, is now required.

There is a growing appreciation that moving towards precision medicine is a more effective approach to patient care, particularly for chronic disease management. Bronchiectasis has a complex underlying aetiology and implementing clinically informative details about patients' airway microbiology, inflammation, remodelling, and respiratory physiology, as exemplified in this study, will ultimately be more beneficial for the development and application of more precise treatment options.

3.6 Acknowledgments

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CHAPTER 4: SCREENING FOR ANTIBIOTIC RESISTANCE USING POOLED-TEMPLATE SHOTGUN METAGENOMICS

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>
> The supplementary information has been included in Appendix 3

4.1 Abstract

Determining the effects of antimicrobial therapies on airway microbiology at a population-level is essential. Such analysis allows, for example, surveillance of antibiotic-induced changes in pathogen prevalence, the emergence and spread of antibiotic resistance, and the transmission of multi-resistant organisms. However, current analytical strategies for understanding these processes are limited. Culture- and PCR-based assays for specific microbes require the a priori selection of targets, while antibiotic sensitivity testing typically provides no insight into either the molecular basis of resistance, or the carriage of resistance determinants by the wider commensal microbiota. Shotgun metagenomic sequencing provides an alternative approach that allows the microbial composition of clinical samples to be described in detail, including the prevalence of resistance genes and virulence traits. While highly informative, the application of metagenomics to large patient cohorts can be prohibitively expensive. **I aimed to develop a novel, cost-effective strategy for screening patient cohorts for changes in resistance gene prevalence.** By combining metagenomic screening of pooled DNA extracts with validatory quantitative PCR-based analysis of candidate markers in individual samples, population-level changes in the relative abundance of specific macrolide resistance genes were identified. This was tested using sputum samples from

a randomised placebo-controlled trial of erythromycin in adults with bronchiectasis and has the potential to provide an important adjunct to current analytical strategies, particularly within the context of antimicrobial clinical trials.

4.2 Introduction

As in all clinical disciplines, the management of patients with chronic respiratory diseases is subject to a process of ongoing refinement, including through the development of novel antimicrobial drugs and treatment strategies. Understanding the impact of antimicrobial treatments for individual recipients allows the personalisation of clinical management. However, determining the effects of treatments at a population level is also crucial, providing a means to predict shifts in the prevalence of respiratory pathogens, or the emergence of antimicrobial resistance, within large patient groups.

The impact that evolving treatment strategies can have on airway microbiology can be seen, for example, in changes in the cystic fibrosis (CF) airway microbiota during recent decades. Within this context, the use of anti-pseudomonal treatments, including parenteral therapies and fluoroquinolones, have been implicated in the emergence of *Stenotrophomonas maltophilia* as an airway pathogen (Burns et al., 1999; Denton et al., 1996). Likewise, increasingly intensive antibiotic use appears to be a contributory factor in the increasing prevalence of non-tuberculous mycobacteria (Bar-On et al., 2015; Catherinot et al., 2013). The impact of antibiotic use is also reflected in the increasing frequency of multi-drug resistant organisms in the airways of patients with chronic respiratory disease, with an estimated 25-45% of adult CF patients chronically infected with multi-drug resistant bacteria (Lechtzin et al., 2006). For example, CF-derived methicillin-resistant *Staphylococcus aureus* (MRSA) isolates increasingly show resistance to newer therapies, including linezolid (Champion et al.,

2014; Hill et al., 2010), ceftaroline (Long et al., 2014) and tigecycline (Champion et al., 2014), presumably as a result of frequent and prolonged exposures (Parkins and Floto, 2015).

Despite the importance of understanding the impact of antimicrobial exposure on the airway microbiome in those with respiratory disease, characterising this process remains challenging. Assessments of antibiotic-associated changes in microbiology are typically limited to a small group of predefined pathogens or resistance genes. The standard analytical approaches employed in clinical antibiotic trials fail to assess major aspects of antibiotic resistance, including shifts in the composition of the wider airway microbiota, and the carriage of transmissible resistance determinants by populations of commensal microbes. The absence of suitable strategies to determine antibiotic impact has resulted in significant gaps in our understanding of how widely employed therapies affect the complex microbiota of the respiratory tract.

Shotgun metagenomic sequencing is an emerging technology that allows highly detailed characterisation of airway microbiota through the analysis of total microbial DNA from clinical samples, including determination of the prevalence of virulence factors and resistance determinants (Rogers et al., 2015). While metagenomic approaches have been shown to be highly effective in describing changes in the microbiome across a wide range of clinical contexts (Wang and Jia, 2016), the cost of its employment within population-scale studies is commonly prohibitive.

This chapter describes a novel, cost-effective, strategy to inform the use of assays for specific resistance genes or microbial taxa, based on deep metagenomic screening of pooled study cohort DNA. The application of this approach is illustrated through the analysis of samples from a previously reported randomised controlled trial of long-term low dose macrolide therapy in adults with bronchiectasis.

4.3 Methods

4.3.1 Study population

The BLESS randomised placebo-controlled trial is described in detail in Chapter 3.3. The analysis reported here was based on paired baseline and week 48 sputum samples from 32 members of the treatment group, and subsequent analysis between treatment group and placebo group subjects (n=32, and n=31, respectively). Patient baseline characteristics are described in Table E3.1 of Appendix 3.

4.3.2 DNA extraction and shot metagenomic sequencing

Sputum was collected as detailed in Chapter 3.3.2, and DNA extracted as detailed in Chapter 2.3.3. DNA extracts were pooled in those receiving erythromycin according to time-point (before or after trial) and subject to microbial DNA enrichment (NEBNext® Microbiome DNA Enrichment Kit). DNA fragmentation was performed using TruSeq Nano DNA Library Prep Kit (Illumina), prior to 150bp paired-end metagenomic shotgun sequencing using an Illumina HiSeq 2500 system at the SA Health and Medical Research Institute, Adelaide. Reads have been uploaded to the NCBI Sequence Read Archive (SRA) under BioProject ID: 397083.

4.3.3 Pooled template sequence bioinformatic processing

Sequences were quality filtered using Trimmomatic v0.32 (Bolger et al., 2014) and human-derived reads removed using BBMap v35.40 (comparing reads to the NCBI human reference genome) (Bushnell, 2016). Contigs were *de novo* assembled using IDBA-UD v1.1.1 (Peng et al., 2012), followed by identification of open-reading frames using MetaGeneMark v3.26 (Besemer and Borodovsky, 2005). Genes were collapsed using CDHit v4.6.6 (Fu et al., 2012) where 39,013 genes with greater than 100 bp were identified and concatenated into a non-redundant gene catalogue. Blast+ v2.6.0 was used to identity antimicrobial resistance genes from the CARD database v1.1.7 (Jia et al., 2017) with an evalue score < $1x10^{-20}$.

Validation of resistance gene assignment was performed by mapping the gene-catalogue to the MEGARes database v1.01. Reads from each pooled sample were then aligned to the gene-catalogue using Soap v2.21 and antimicrobial resistance genes quantified. Sample counts were normalised to counts per million (CPM) total reads and change in CPM (ΔCPM) was calculated by $CPM_{post\ treatment} - CPM_{pre\ treatment}$.

4.3.4 Validation of pooled-template process with qPCR

Specific resistance genes that were identified as associated with erythromycin treatment through metagenomics were subsequently quantified in DNA extracts from individual sputum samples by qPCR. Previously published assays were used for *erm*(A) (Jung et al., 2009), *erm*(B) (Zhang et al., 2011), *erm*(C) (Martineau et al., 2000), 16S (Nadkarni et al., 2002), and *smp*(B) (Reddington et al., 2015) genes. Primers for quantification of the multi-drug efflux gene, *hmrM*, were designed within this study (see supplement of Appendix 3). For analysis of qPCR results, Wilcoxon rank tests were performed on fold change normalised to 16S copy number to compare erythromycin paired samples to placebo control paired samples (n=31 pairs).

4.4 Results

4.4.1 Resistance gene carriage from pooled-template shotgun metagenomic analysis

A schematic of the pooled-template metagenomic sequencing strategy, and subsequent qPCR-based validation, is shown in Figure 4.1. Following removal of low-quality reads and human DNA (approximately 90% of total read depth), a mean sample read depth of 12,866,780 was achieved. Approximately half a million reads have been previously reported to analyse the microbial composition in individual sputum samples (Moran Losada et al., 2016). Mapping of sequence reads to the CARD database resulted in the detection of a total of 102 resistance-

associated genetic determinants. The distribution of normalised reads that mapped to the CARD database in pre- and post-trial pooled samples is shown in Figure 4.2. Detected genes represented a range of resistance mechanisms, including antibiotic inactivating enzymes, efflux pumps, and effector site protection proteins, and conferred resistance to a number of antibiotic classes, including aminoglycosides, beta-lactams, glycopeptides, and tetracyclines.

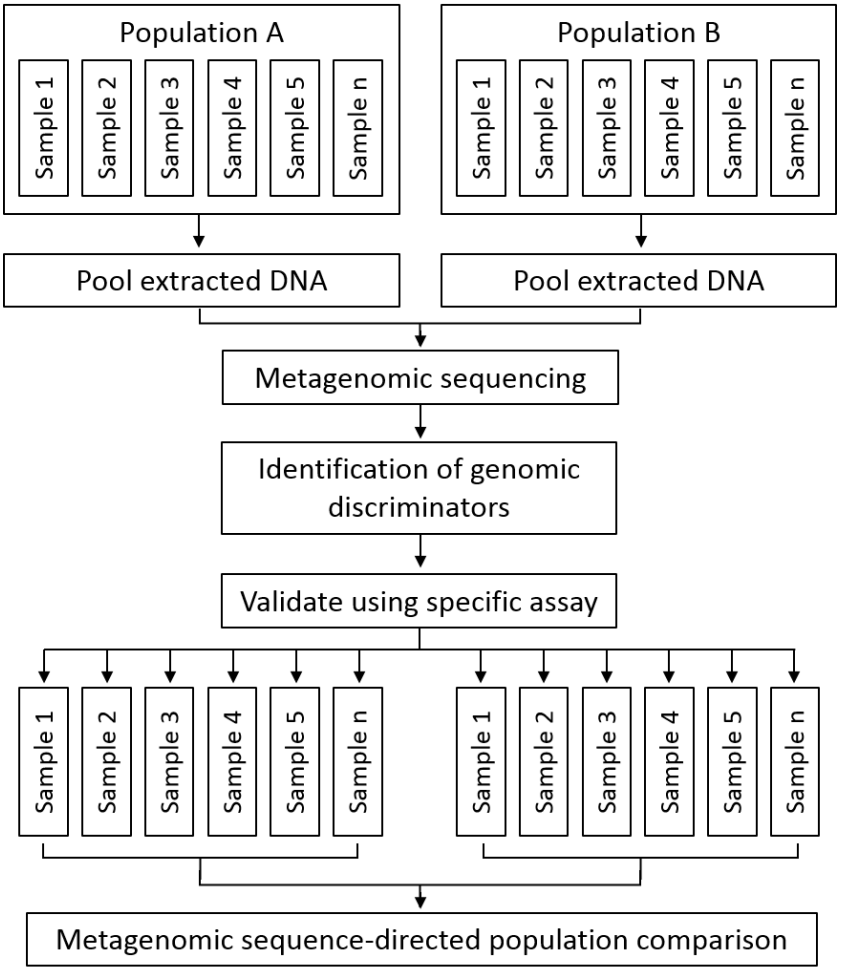

Figure 4.1: Principle of pooled-template metagenomic sequencing. Sample DNA extracts from a population of interest are pooled together according to a pre-specified variable of interest (such as treatment or time-point). Metagenomic sequencing is then performed on pooled samples and regions that discriminate between populations are determined. Targeted assays (such as qPCR) are then performed on individual samples for gene specific enumeration.

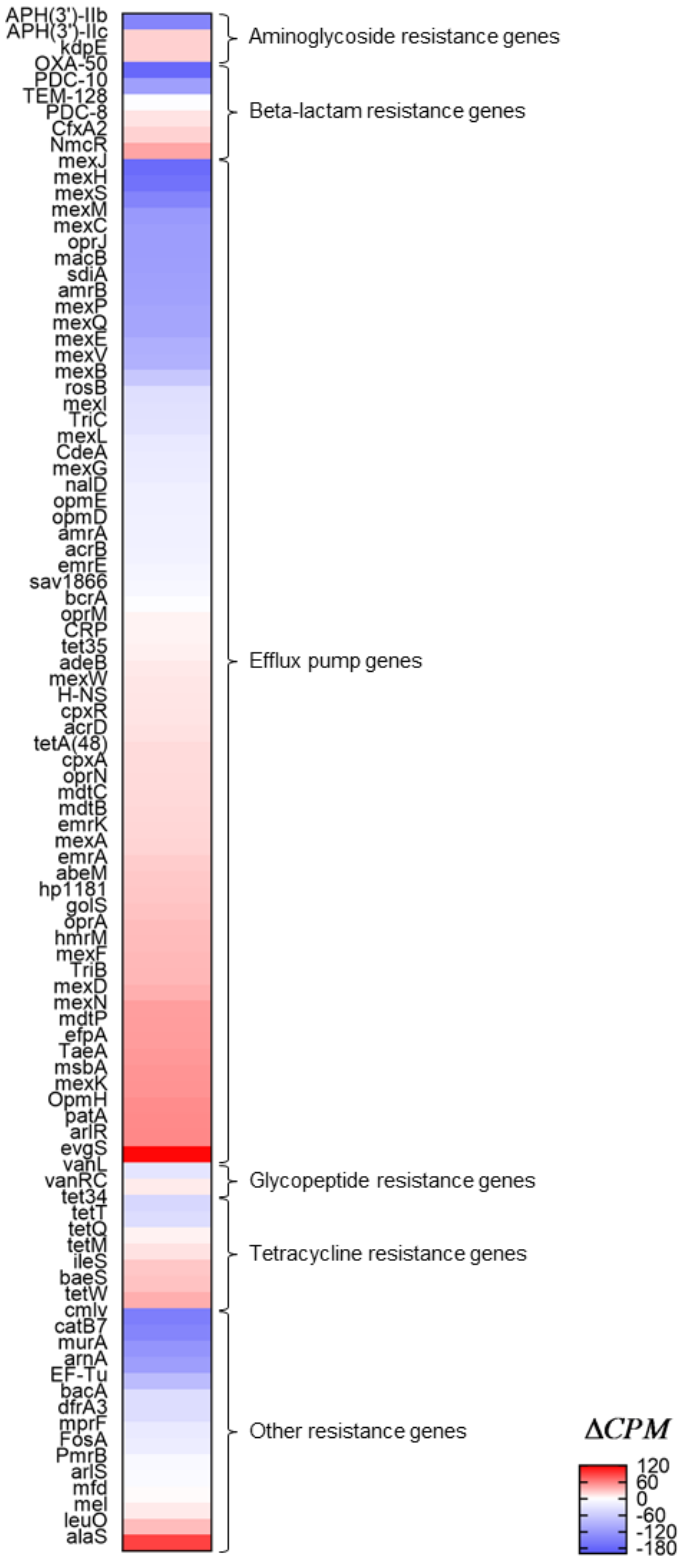

Figure 4.2: Resistome of pooled-template sputum before and after erythromycin therapy. Counts per million total reads (CPM) of major antibiotic resistance genes identified by the comprehensive antibiotic resistance gene database (CARD). Positive ΔCPM (red) indicates higher in samples post erythromycin. Resistance genes grouped by function, as defined by CARD.

4.4.2 Validation of chromosomal resistance gene carriage

A substantial proportion of the genes identified through resistome analysis were chromosomally-encoded, non-transmissible, resistance determinants. Changes in the level of carriage of these genes during the trial therefore reflected shifts in the relative abundance of the species in whose genomes they are encoded, rather than resistance gene acquisition or loss. For example, the multidrug efflux pump gene, *hmrM*, appeared to increase in response to erythromycin therapy. This gene is chromosomally-encoded by *H. influenzae* however, and subsequent qPCR analysis revealed *hmrM* levels to be correlated with *H. influenzae* levels ($r=0.74$, $p<0.001$, Figure 4.3). The observed increase in prevalence of *hmrM* is therefore likely to simply reflect an increase in the relative abundance in *H. influenzae* in the assessed patient group (a median increase of $1.4x10^3$ copies was observed between pre- and post-erythromycin samples). This phenomenon could explain apparent changes in the group-level abundance of other chromosomally-encoded resistance genes, such as an observed decrease in the relative abundance of *pat*(A), a chromosomally-encoded fluoroquinolone resistance gene carried by *Streptococcus pneumoniae* (El Garch et al., 2010), and *aph*(3')-IIb, a chromosomally-encoded aminoglycoside resistance gene carried by *P. aeruginosa* (Stover et al., 2000).

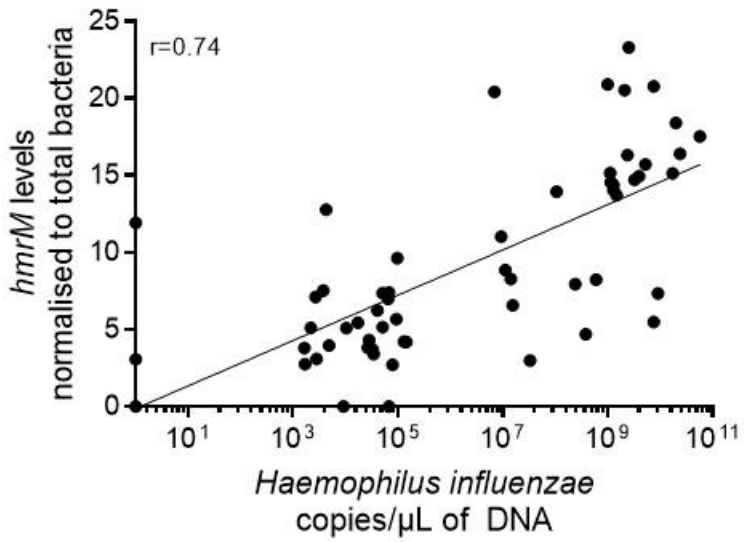

Figure 4.3: Correlation between *hmrM* and *Haemophilus influenzae*. *hmrM* (normalised to total bacteria) against *H. influenzae* copy number (determined by comparing to known standard curve). Significance determined by Spearman's rank order correlation.

4.4.3 Quantification of transmissible resistance genes

Several of the resistance genes identified through pooled-template metagenomic sequencing were encoded on mobile genetic elements and have been shown previously to be transmissible between bacterial species. These include a number of transmissible genetic elements that confer resistance to a range of antibiotics. However, the key plasmid-encoded erythromycin resistance methylase gene, *erm*(B), which has previously been shown to increase with macrolide treatment, was not detected. To determine whether this gene was present in samples but not detected, or whether it was not present, *erm*(B) specific qPCR was performed on samples. 92% of patients had detectable levels of *erm*(B) and there was a significant increase in the relative abundance of *erm*(B) in subjects who received erythromycin (p=0.006), but not in those who received placebo (p=0.065, Figure 4.4). In contrast, other transmissible macrolide-resistance determinants were shown by follow-up qPCR analysis to not contribute

substantially to the post-trial resistome. For example, *erm*(A), a resistance gene found in staphylococci (Ghanbari et al., 2016), was present in only four subjects (two in the treatment group and two in the control group). The *erm*(C) resistance determinant, which is also found in staphylococci (Ghanbari et al., 2016), was detected more frequently (68% of subjects receiving placebo and 81% of subjects receiving erythromycin), however, *erm*(C) levels did not change significantly over the course of the trial. The rates of carriage of *erm*(A) and *erm*(C) are consistent with those reported in *S. aureus* clinical isolates more widely (Aktas et al., 2007; Ghanbari et al., 2016).

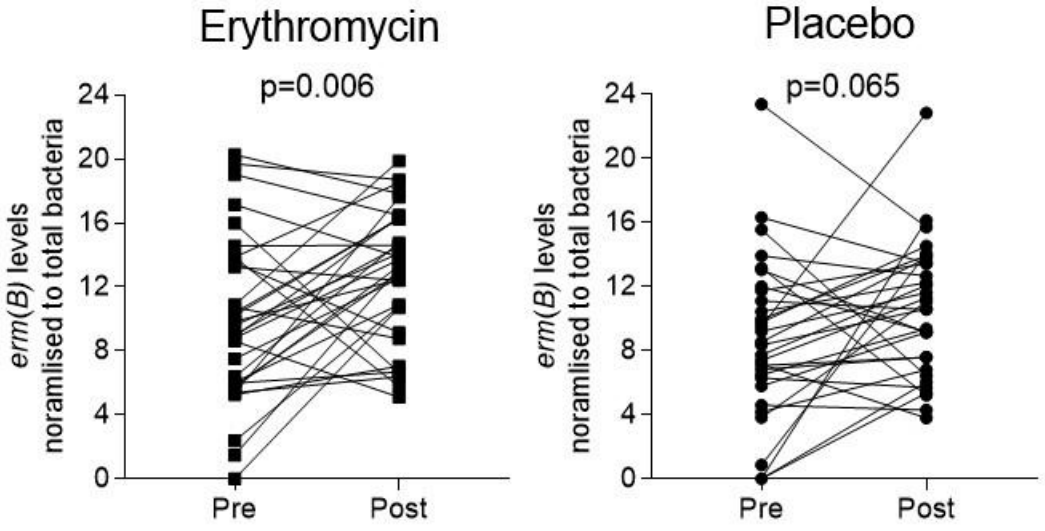

Figure 4.4: Changes to *erm*(B) levels in erythromycin and placebo groups. Paired sample analysis of *erm*(B) (normalised to total bacteria). Significance determined by Wilcoxon signed-rank test.

4.5 Discussion

This chapter describes a cost-effective approach that can be used to guide the assessment of changes in antibiotic resistance gene carriage, which might represent a useful adjunct to conventional approaches that are based on *a priori* target selection. As an illustration, the BLESS randomised placebo-controlled trial that preceded this study included an

assessment of whether erythromycin therapy resulted in an increased relative abundance of macrolide resistant oropharyngeal streptococci using culture-based proportional sensitivity testing (Serisier et al., 2013). While this narrow analysis identified a significant increase in the proportion of macrolide-resistant streptococci, neither the level of transmissible resistance gene carriage in non-streptococcal species, nor the molecular basis of resistance, were determined. The use of pooled metagenomic sequencing revealed a number of resistance determinants for follow-up analysis where targeted qPCR assays were subsequently applied to DNA extracts from individual samples. This validation step confirmed significant increases in the abundance of, for example, the transmissible macrolide resistance gene, *erm*(B), in patients receiving erythromycin.

By pooling sample DNA at the pre-sequencing, rather than the post-sequencing, library-construction stage (as performed in standard metagenomic sequencing approaches), the cost of the analysis is calculated to be approximately 15% of that required to analyse all of the samples individually (although precise costs will be influenced by sample number, processing methodologies, and desired sequencing depth). However, despite this substantial reduction in expense, it is important to be aware of some of the limitations that are inherent in this approach. For example, variations in bacterial load between samples from different patients mean that pooling DNA based on total concentration could result in the contribution of individual samples to meta-microbiome characteristics being unequal. In addition, the non-normal distribution of microbiome traits within a population could lead to the identification of traits as potential inter-group discriminators based on their particularly high abundance in a small number of individuals (although the impact of this effect is likely to decrease with increasing cohort size).

A limitation of all metagenomic sequencing is the challenge to differentiate between changes in the carriage of resistance determinants due to direct selective pressure versus changes in resistance gene carriage, because of shifts in the relative abundance of the bacterial

populations that encode them. Further, detection of resistance genes may not confer a resistance phenotype. Resistance genes may not be expressed or if expressed it may be at low levels. The resistance genes may also be harboured by organisms that have no clinical importance and, if chromosomally encoded, may not contribute to pathogenesis or clinically relevant community-resistance. Due to such limitations, the approach described should be used as an additional means to identify markers for further analysis, rather than as a means to characterise antibiotic associated effects on airway microbiology in itself.

As an illustration of the potential of the pooled-template metagenomic analysis, shifts in the airway resistome were examined. This application targeted the global health concern of monitoring of antibiotic resistance. Patients with chronic lung diseases have an increased exposure to antibiotics, with the emergence of resistance correlating closely with consumption (Goossens, 2009). The resistome associated with the airway microbiota in these patients is likely to be a substantial contributor to the emergence and expansion of populations of multi-resistant organisms (Sherrard et al., 2014) and their potential transmission to individuals within the wider community. However, despite its application to the assessment of the airway resistome here, pooled-template metagenomic analysis can be applied equally to assessment of species distribution (Truong et al., 2015), or to identify changes in community level carriage of pathogenicity traits (for example, through alignment to virulence factor genetic databases). By aligning regions that encode antibiotic binding sites, it may also be possible to determine the relative abundance of resistance-conferring single nucleotide polymorphisms (SNPs). Obtaining such information could provide important clinical insight. For example, while *de novo* mutations in the 23S rRNA are the principal cause of macrolide resistance in non-tuberculous mycobacteria (Brown-Elliott et al., 2012), relatively little is known currently about the dynamics of their emergence during macrolide therapy.

The costs of metagenomic sequencing, and the associated costs of high performance computing required for bioinformatic analysis, are likely to continue to fall. However, by providing a low-cost means to perform unbiased surveys of large patient cohorts, strategies such as the one described here represent a useful means of identifying potentially important discriminatory microbiome features for follow-up analysis.

4.6 Acknowledgements

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4.7 References

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CHAPTER 5: THE IMPACT OF AZITHROMYCIN ON LUNG MICROBIOTA AND RESISTOME IN SEVERE ASTHMA

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>
> The supplementary information has been included in Appendix 4

5.1 Abstract

The macrolide drug, azithromycin, reduces exacerbations in adults with persistent symptomatic asthma. Owing to the pleotropic properties of azithromycin, unintended bacteriological consequences such as augmented pathogen colonisation or spread of antibiotic-resistance organisms can occur, questioning the long-term safety of azithromycin maintenance therapy. **I aimed to assess the effect of azithromycin on the airway microbiome, including the abundance of known airway pathogens, and carriage of antibiotic-resistance genes in persistent symptomatic asthma.**

16S rRNA gene sequencing and quantitative PCR was performed on induced sputum samples from participants of the AMAZES trial to assess the effect of azithromycin on microbiology. Pooled-template shotgun metagenomic sequencing, quantitative PCR, and culture isolate whole genome sequencing were performed to assess the effect of azithromycin of antibiotic resistance gene carriage.

Paired sputum samples were available from 61 patients, 34 in the placebo group and 27 in the azithromycin group. Azithromycin did not affect the total number of bacteria in the lower airways (p=0.37). However, azithromycin was associated with a significant decrease in the

total diversity of bacterial taxa (p=0.026), and a significant decline in *Haemophilus influenzae* load (p<0.001). A significant increase in macrolide-resistant organisms and the carriage of antibiotic resistance genes were also observed, including the macrolide resistance genes (*erm*(B), *erm*(F), *msr*(E), *mef*(A), and *mel*) and tetracycline resistance genes (*tet*(M) and *tet*(W)).

In patients with persistent uncontrolled asthma, azithromycin as an add-on therapy did not result in a shift in the prevalence of pathogenic organisms such as *Pseudomonas aeruginosa*, as found in the analysis in other chronic respiratory diseases. However, the findings of this study highlight the need to examine antibiotic resistance carriage in patients receiving azithromycin and advocate for further research into the efficacy of non-antibiotic macrolides.

5.2 Introduction

Asthma is a global health issue affecting an estimated 235 million people worldwide (World Health Organisation, 2017). For the majority of patients, standard corticosteroids and bronchodilator therapies manage symptoms effectively. However, for an estimated 50 million patients, asthma is characterised as persistent and uncontrolled (Peters et al., 2006). These patients experience more severe airway inflammation (Chung et al., 2014), exhibit an altered airway microbiology (Green et al., 2014; Taylor et al., 2018), and, importantly, are at a higher risk of severe exacerbations, which disproportionately contributes to asthma-associated hospitalisations and healthcare costs (Calhoun et al., 2014; Ivanova et al., 2012; Wang et al., 2010). Recently, long-term azithromycin therapy was shown to reduce exacerbations and improve quality of life in this patient population (Gibson et al., 2017), signifying azithromycin therapy as a valuable addition to existing regimens for treating asthma (Brusselle and Pavord, 2017). However, though clinical side-effects from azithromycin were minimal, the potential

for detrimental consequences of long-term therapy on airway microbiology remains poorly understood.

Azithromycin is a member of the macrolide drug class that can inhibit bacterial protein synthesis (Hansen et al., 2002), reduce pathogen virulence (Imperi et al., 2014) and also modulate multiple pathways of host immunity (Altenburg et al., 2011; Gielen et al., 2010; Simpson et al., 2008). The use of macrolides has therefore been found to alter the composition of bacteria in the airways (Choo et al., 2018; Rogers et al., 2014; Slater et al., 2014), which can have negative impacts, such as promoting growth of pathogenic organisms. In bronchiectasis, for example, 48 week treatment with erythromycin (another macrolide drug) has been shown to increase the relative abundance of the airway pathogen *Pseudomonas aeruginosa* in the lungs (Rogers et al., 2014). In persistent uncontrolled asthma, the lower airway microbiota is associated with multiple markers of asthma severity including lower lung function (Green et al., 2014), neutrophilic inflammation (Taylor et al., 2018), and corticosteroid resistance (Goleva et al., 2013), however the effect of azithromycin on the airway microbiota in these patients is largely unknown.

Along with selection of airway pathogens, the administration of macrolides has been previously reported to increase the carriage of macrolide-resistant bacteria (Altenburg et al., 2013; Brusselle et al., 2013; Choo et al., 2018; Kastner and Guggenbichler, 2001; Malhotra-Kumar et al., 2007; Mustafa et al., 2017; Saiman et al., 2010; Serisier et al., 2013). Macrolide resistance can be acquired in bacteria intrinsically, through genetic mutations, or extrinsically, through acquisition of transmissible macrolide resistance genes from an existing macrolide resistant organism. Acquired macrolide resistance is a global health concern, particularly relating to the treatment of infections where macrolides are routinely prescribed, such as in nontuberculous mycobacterium infections (Egelund et al., 2015), and atypical infections in community acquired pneumonia (Waterer et al., 2011) and sexually transmitted infections

(Workowski et al., 2015). Identifying reservoirs of transmissible resistance genes is a global health strategy to limit the dissemination of antibiotic resistance to the wider community, however this has not been investigated in the patients receiving azithromycin in asthma.

In this secondary analysis from the Asthma and Macrolides: The Azithromycin Efficacy and Safety (AMAZES) study, undirected approaches were used to assess whether azithromycin affects the abundance of opportunistic pathogens and carriage of antibiotic resistance genes in the airways of patients with persistent uncontrolled asthma.

5.3 Methods

5.3.1 Study population

AMAZES was a 12-month, double-blind, randomised, placebo-controlled trial (ACTRN12609000197235) to determine whether oral azithromycin decreases the frequency of asthma exacerbations in adults (≥18 years) with symptomatic asthma despite current use of inhaled corticosteroid and long-acting bronchodilator, and who had no hearing impairment or abnormal prolongation of the corrected QT interval (Gibson et al., 2017). Patients were randomly assigned (1:1) to receive azithromycin 500 mg or placebo three times per week for 48 weeks. Of the 420 patients recruited for the trial, sputum samples from 61 patients were analysed here based on available stored sputum samples. Inclusion and exclusion criteria are described in section 2.3.1 and Appendix 1.

5.3.2 Sputum collection and DNA extraction

Induced sputum was collected at baseline and after 48 weeks of the trial, as described in Chapter 2.3. Nucleic acid extraction was performed on frozen sputum samples as described in Chapter 2.3, using a combined physical, enzymatic, and heat-based cell lysis, followed by

phenol-chloroform extraction and DNA recovery using EZ-10 Spin columns (Bio Basic, Inc., Ontario, Canada).

5.3.3 16S rRNA gene amplicon sequencing and shotgun metagenomic sequencing

The V1-3 hypervariable region of the bacterial 16S rRNA gene was sequenced as described in Chapter 2.3. Shotgun metagenomic sequencing was performed on DNA extracts pooled by treatment group and time point as described in Chapter 4.3 with minor changes. Pooled template DNA was sequenced on an Illumina HiSeq 4000 with paired-end 150bp reads. Sequencing data are deposited in the European Nucleotide Archive database under PRJEB26356 for 16S sequences and PRJEB27079 for shotgun metagenomic sequences.

5.3.4 Bioinformatic processing

16S amplicon sequencing reads were processed using QIIME v1.9.1 and assigned taxonomy using the SILVA database v1.23 as a reference. Sequence data were subsampled to 1,833 reads. Two samples from the azithromycin group did not reach this threshold and were removed. Microbiome diversity metrics (Faith's phylogenetic diversity and UniFrac distances) were computed using QIIME v1.9.1.

Metagenomic sequences were processed as described in Chapter 4.3. Quality filtering and removal of human DNA yielded an average of 6,561,275 reads per sample (Table 5.1). Reads were *de novo* assembled (Peng et al., 2012) and 50,361 genes with greater than 100 bp were identified and concatenated into a non-redundant gene catalogue. Screening of antibiotic resistance genes was performed by mapping genes to the comprehensive antibiotic resistance database (CARD) v1.1.7 (Jia et al., 2017) and sample counts were normalised to ΔCPM.

Table 5.1: Summary of pooled sample metagenomic reads

	Total reads	Quality human reads	Quality non-human reads
Azithromycin pre	112,860,634	92,617,788	6,135,414
Azithromycin post	130,838,966	107,280,206	5,647,466
Placebo pre	153,440,890	122,872,560	7,170,758
Placebo post	148,938,168	126,622,016	7,291,460

5.3.5 Resistance gene and bacterial species quantification

Levels of resistance genes *erm*(B), *erm*(F), *mel*, *msr*(E), *tet*(M) *and tet*(W) were assessed using SYBR Green assays, and the *mef* gene (detecting both *mef*(A) and *mef*(E)) was assessed using a Taqman assay based on previously described primer pairs (Table 5.2). Levels of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* were quantified using SYBR Green assays and *Haemophilus influenzae* and *Staphylococcus aureus* were quantified using Taqman assays based on previously described primer pairs (Table 5.3).

For SYBR Green qPCR assays, 1 µL of DNA extract, 0.2 µM of each primer, 17.5 µL of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, USA) and the appropriate volume of water was added to a 35 µL total reaction volume. For Taqman qPCR assays, 1 µL of DNA extract, 0.2 µM of each primer, 0.1 µM of target probe, 17.5 µL of 2X KAPA Probe Fast qPCR Master Mix (KAPA Biosystems Inc., Wilmington, USA) and the appropriate volume of water was added to a 35 µL total reaction volume. Quantitative real-time PCR assays were performed on three technical replicates, at a 10 µL reaction volume per replicate, on a QuantStudio 6 and 7 Flex Real-Time PCR system (Applied Biosystems, Carlsbad, USA). Mean cycle threshold (Ct) of resistance gene replicates were normalised to 16S mean Ct to give ΔCt. ΔCt_{max}-ΔCt was calculated so that a higher value represents higher gene carriage. Total bacteria (as determined by 16S quantification) and specific bacterial species were quantified to a copy number per µL of extracted DNA (copies/µL of DNA) by comparing the sample Ct to a standard curve with known copy numbers.

Table 5.2: Primers used to quantify antibiotic resistance gene carriage

Target gene	**Sequence (5' - 3')**	**Amplicon size (bp)**	**Reference**
erm(B)	5'- GAAAGCCRTGCGTCTGACATC 5'- CGAGACTTGAGTGTGCAAGAGC	105	(Zhang et al., 2011)
erm(F)	5'-CGGGTCAGCACTTTACTATTG 5'-GGACCTACCTCATAGACAAG	466	(Choo et al., 2018; Chung et al., 1999)
mef	5'-TATGGAGCTACCTGTCTGGA 5'-GGTACTAAAAGTGGCGTAACC HEX-CCGTAGCATTGGAACAGCTTTTC-BHQ1	85	(Srinivasan et al., 2011)
mel	5'- GAACGTAAGAGCCAAGCTGCA 5'- GGCACGTTCCGCAATAAATT	51	(Ambrose et al., 2005)
msr(E)	5'- TCGATACGAAGAGGCGATGC 5'- CTTCTGTTTGGTGCCGGTTG	163	This study
tet(M)	5'- CAGAATTAGGAAGCGTGGACAA 5'- CCTCTCTGACGTTCTAAAAGCGTAT	67	(Florez et al., 2014)
tet(W)	5'- GAGAGCCTGCTATATGCCAGC 5'- GGGCGTATCCACAATGTTAAC	168	(Tao et al., 2014)
16S	5'-TCCTACGGGAGGCAGCAGT 5'-GGACTACCAGGGTATCTAATCCTGTT	466	(Nadkarni et al., 2002)

Table 5.3: Primers used to quantify bacterial species levels

Organism	**Sequence (5' - 3')**	**Amplicon size (bp)**	**Reference**
Haemophilus influenzae	5'- ATTAAATGTTGCATCAACGC 5'- GACTTTTGCCCACGCAC FAM-ACGRTTTTACCATAGTTGCACTTTCTC-BHQ	140	(Corris et al., 2015)
S. pneumoniae/ S. pseudo-pneumoniae	5'-GTGCTITGAAATTCTATGCTTC 5'-GTGGAGCTACCTTATTTTTTAC	135	(Sistek et al., 2012)
Staphylococcus aureus	5'-AAATTACATAAAGAACCTGCGACA 5'-GAATGTCATTGGTTGACCTTTGTA FAM-AATTTAACCGTATCACCATCAATCGCTTT-BHQ1	87	(Thomas et al., 2007)
Pseudomonas aeruginosa	5'-CGAGTACAACATGGCTCTGG 5'-ACCGGACGCTCTTTACCATA	117	(Feizabadi et al., 2010)

5.3.6 Bacterial culture and whole genome sequencing

Sputum was diluted and plated on horse blood agar (HBA), Choc-B agar and chromID™ *S. aureus* Elite agar (SAIDE) plates to isolate streptococci, *Haemophilus* species and *Staphylococcus aureus*, respectively. Plates were cultured for 18-48 h at 35 °C and with 5% CO_2 (for HBA, Choc-B plates). Individual colonies were subcultured and identified by Matrix Assisted Laser Desorption/Ionization-time of flight mass spectrometry (MALDI-TOF), using a Bruker Microflex MS score ≥2.0. Identification of *S. pneumoniae* was determined by optochin sensitivity. Azithromycin sensitivity was determined by disk-diffusion according to EUCAST protocols (Matuschek et al., 2014) and using EUCAST Clinical Breakpoints from v. 8.1. This is the most current version and states the clinical breakpoint for *Haemophilus* is conflicting and should be guided by the epidemiological cut-offs. *Haemophilus* breakpoint sensitivity to azithromycin was therefore determined by comparing inhibition zones to that of macrolide-sensitive *H. influenzae* NTCC8468.

Following disk-diffusion testing, single isolate bacterial lawns were then suspended at approximately 5 MacFarland, in 10% glycerol/saline solution and stored at -80 °C. DNA extraction was performed on 50 µL of bacterial solution using a modified AxyPrep Mag Tissue-Blood gDNA Purification kit protocol (Corning, Big Flats, NY, USA), with an additional 2.9 mg/ml lysozyme and 0.14 mg/ml lysostaphin (Sigma-Aldrich, St. Louis, MO, USA) 1 h at 37 °C incubation for gram positive bacteria. Bacterial DNA was processed for whole genome sequencing (WGS) using Illumina Nextera XT DNA Library Preparation Kit according to preparation guidelines. The libraries were normalised to 2 nM and sequenced on an Illumina NextSeq 550. Sequences were *de novo* assembled using the Nullarbor pipeline v20170519 and antibiotic resistance gene carriage was identified from predicted protein sequences using Blast+ v2.6.0 against the CARD database v1.1.3 with an evalue score $<1x10^{-20}$.

5.3.7 Statistical analysis

All continuous data were tested for normality using GraphPad Prism v7.03. Unpaired parametric data were analysed by Student's t-test, unpaired non-parametric data were analysed by the Mann Whitney U test, paired non-parametric data were analysed by the Wilcoxon matched-pairs signed rank test, and proportional data were analysed by Fisher's exact test. Multivariate analysis (PERMANOVA) of 16S rRNA taxonomic profiles was performed using the PRIMER 6 software (PRIMER-E Ltd, Plymouth, UK). 16S rRNA taxonomic changes were analysed by linear discriminant analysis effect size (LEfSe) with alpha=0.05 and a "one vs all" test (Segata et al., 2011).

5.4 Results

5.4.1 Baseline demographics

The AMAZES study took place between June 12, 2009, and Jan 31, 2015 where adults with persistent uncontrolled asthma (showing evidence of variable airflow obstruction and loss of asthma control (asthma control score (ACQ6) ≥0.75) despite treatment with maintenance ICS or LABA) were randomly assigned to either 500 mg of azithromycin or placebo, three times per week for 48 weeks (Gibson et al., 2017). Of these participants, raw sputum was available at both baseline and after 48 weeks from 27 patients who received azithromycin and 34 who received placebo. There were no significantly different clinical characteristics or sputum microbiological measures at baseline between groups (Table 5.4), although asthma duration and smoking history trended towards a difference. There were also no significant differences in baseline sputum microbial community structure (p=0.780, pseudo-F=0.845).

5.4.2 Azithromycin therapy reduces sputum phylogenetic diversity

Paired comparisons of sputum between baseline and after 48 weeks of treatment indicated that azithromycin was not associated with a significant change in total bacterial load ($p=0.37$, Figure 5.1A). However, bacterial diversity was significantly reduced in the azithromycin group (Faith's phylogenetic, $p=0.026$, Figure 5.1B) but not in the placebo group ($p=0.47$, Figure E4.1 of Appendix 4). Pairwise PERMANOVA testing identified a significant difference in microbiome composition in the azithromycin group ($p=0.014$, $t=1.30$), which was not seen in the placebo group ($p=0.988$, $t=0.827$). This change was also reflected in higher unweighted UniFrac distance in the azithromycin group compared to the placebo group ($p=0.022$, Figure 5.1C). Together, these results demonstrate that, in the lower airways, azithromycin therapy reduces bacterial diversity while total bacterial levels remain.

Table 5.4: Characteristics of patients at baseline

	Placebo (n=34)	Azithromycin (n=27)	p-value
Age, yrs[¥]	57.7 (17.4)	59.4 (12.5)	0.682
Age asthma diagnosis, yrs[Ω]	9.50 (5.0-27.3)	23.0 (6.0-55.0)	0.125
Asthma duration[Ω]	41.8 (24.0-56.1)	30.2 (13.3-45.5)	0.070
Female[ϒ]	12 (35.3)	15 (55.6)	0.129
Height, cm[¥]	168.2 (9.7)	167.2 (9.8)	0.699
Weight, kg[Ω]	86.6 (71.6-99.7)	83.5 (74.1-96.7)	0.684
Atopy[ϒ]	31 (93.9)	21 (77.8)	0.124
Ever-smoker[ϒ]	9 (26.5)	8 (29.6)	0.999
Pack years, yrs [Ω]	6.0 (1.0-9.4)	25.8(4.3-46.8)	0.059
ACQ score [¥]	1.72 (0.81)	1.65 (0.91)	0.775
Medications			
ICS dose, BDP mcg/day [Ω]	1000 (800-2000)	900 (800-2000)	0.371
ICS/Long-acting beta agonist[ϒ]	32 (100)	22 (91.7)	0.179
Leukotriene modifier[ϒ]	1 (3.1)	0	>0.999
Long-acting anti-muscarinic[ϒ]	6 (18.8)	7 (29.2)	0.524
Theophylline[ϒ]	0	2 (8.3)	0.179
Oral corticosteroid[ϒ]	0	1 (4.2)	0.429
Pre B2 spirometry [¥]			
Pre B2 FEV_1%	71.1 (16.6)	69.2 (17.0)	0.674
Pre B2 FVC%	81.4 (15.2)	82.3 (12.2)	0.813
Pre B2 FEV_1/FVC%	66.8 (9.2)	66.0 (12.0)	0.751
Blood eosinophils ($\times 10^9$) per L [Ω]	0.3 (0.2-0.4)	0.3 (0.2-0.5)	0.585
Sputum cell counts[Ω]			
Total cell count ($\times 10^6$) per mL	5.8 (2.7-9.8)	4.7 (2.7-7.2)	0.637
Neutrophils (%)	36.8 (19.3-61.0)	37.5 (11.3-58.0)	0.794
Eosinophils (%)	1.75 (0.75-4.75)	2.0 (0.5-7.75)	0.741
Sputum phenotype[ϒ]			
Eosinophilic	13 (38.2)	11 (44.0)	0.862
Neutrophilic	7 (20.6)	3 (12.0)	0.321
Paucigranulocytic	12 (35.3)	10 (40.0)	0.888
Mixed	2 (5.9)	1 (4.0)	0.696
Sputum bacterial count ($\times 10^5$) per mcL of DNA[Ω]	5.66 (1.49-11.67)	3.71 (1.08-9.98)	0.423
Sputum microbiota diversity [Ω]			
Faith's phylogenetic diversity	11.48 (9.39-12.7)	11.16 (8.45-12.8)*	0.547
Simpson's evenness	0.117 (0.065-0.145)	0.117 (0.09-0.167)*	0.423
Shannon-Wiener diversity	5.66 (4.95-6.11)	5.60 (4.79-6.14)*	0.885
Taxa richness	56 (41.75-62)	52 (37.5-67.5)*	0.645

[¥] Mean (SD), Student's t-test; [Ω] Median (IQR), Mann Whitney U test; [ϒ] n(%), Fisher's exact test; *Data available for 25 participants

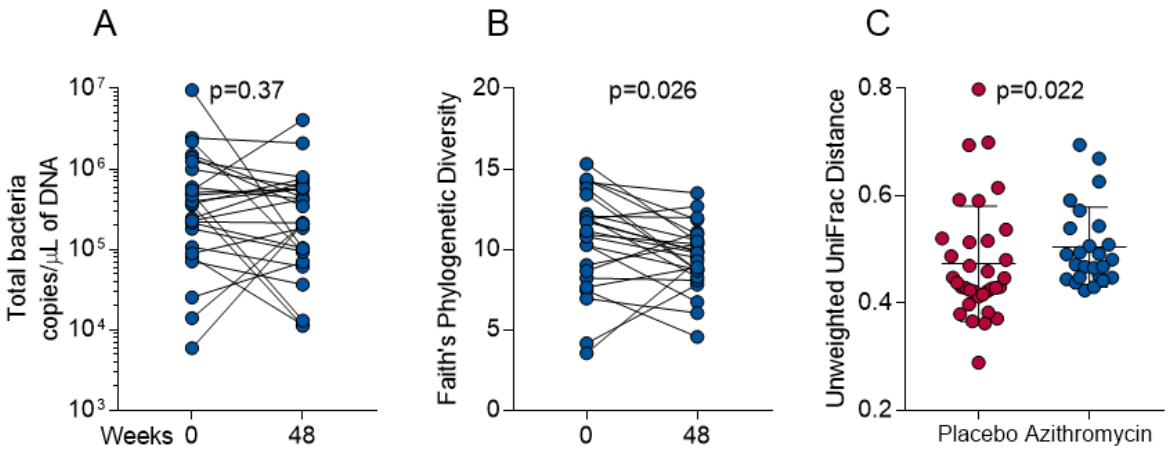

Figure 5.1: Effect of azithromycin on sputum microbiota composition. A) Total bacterial load before and after azithromycin. B) Faith's phylogenetic diversity before and after azithromycin. C) Unweighted UniFrac distance in placebo and azithromycin groups, where a higher dissimilarity distance indicates greater change in microbiota composition between samples before and after placebo and azithromycin.

5.4.3 Azithromycin therapy reduces *Haemophilus influenzae* load

Of all the taxa identified in the lower airways, Gammaproteobacteria had the greatest reduction following azithromycin (p=0.039, log LDA score=4.68, Figure 5.2A), but not placebo (Figure E4.2 of Appendix 4). A range of species belong to Gammaproteobacteria, of which, the absolute levels of the notable opportunistic respiratory pathogen, *H. influenzae*, fell significantly in patients who received azithromycin (p<0.0001), but not placebo (p=0.62, Figure 5.2B). Other lower airway pathogens including *S. pneumoniae*, *S. aureus*, and *P. aeruginosa* appeared unaffected by azithromycin (Figure E4.3 of Appendix 4).

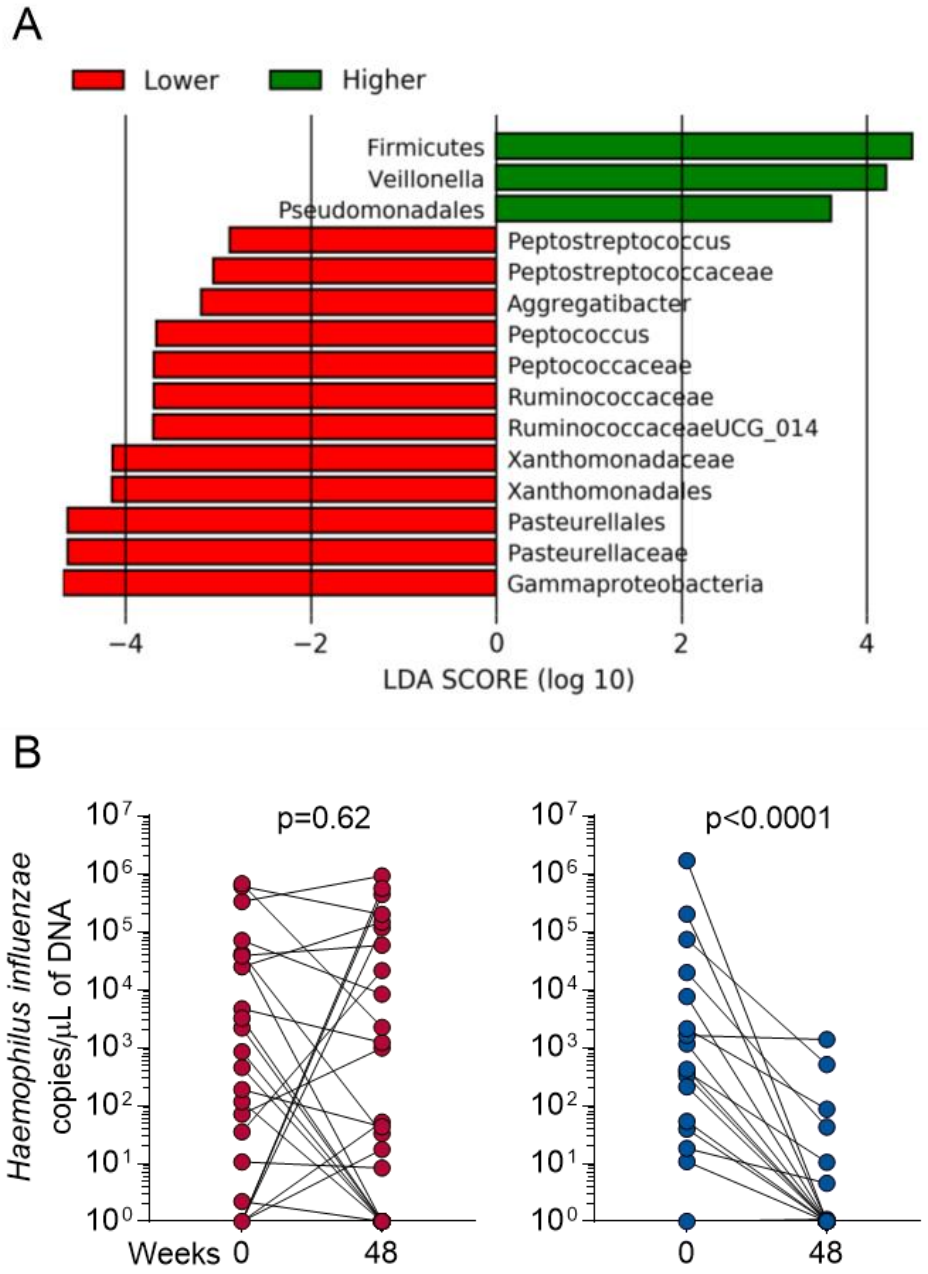

Figure 5.2: Effect of azithromycin on individual taxa. A) Linear discriminant analysis effect size (LEfSe) of taxa that significantly changed in relative abundance following azithromycin. Red indicates taxa that were lower, and green indicates taxa that were higher following azithromycin B) *Haemophilus influenzae* copy number before and after either placebo (left) or azithromycin (right).

5.4.4 Azithromycin therapy increases carriage of antibiotic resistance genes

Carriage of antibiotic resistance genes was screened for by pooled template shotgun metagenomic analysis. Out of the 89 antibiotic resistance genes detected, seven transmissible

genes (*tet*(W), *mel*, *msr*(E), *tet*(M), *erm*(F), *mef*(A), and *erm*(B)) were more highly represented after azithromycin but not placebo (Figure 5.3). This effect was confirmed by qPCR, with all seven resistance genes significantly increased following azithromycin (Figure 5.4) but not placebo (Figure E4.4 of Appendix 4).

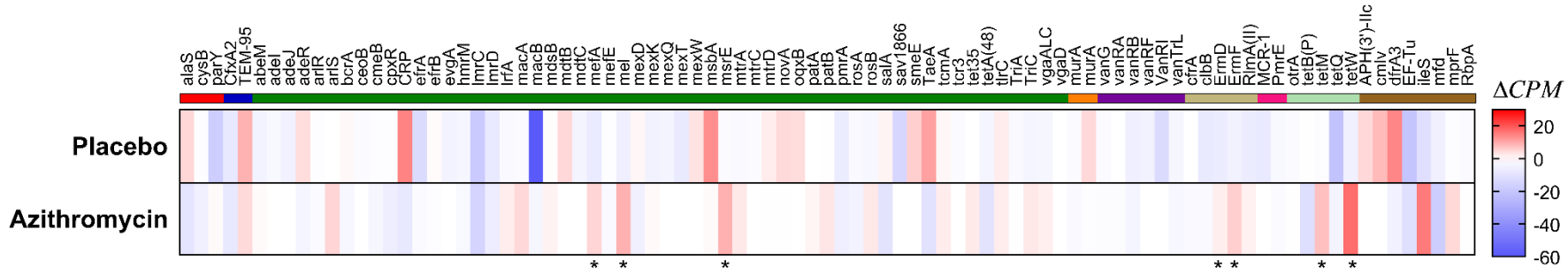

Figure 5.3: Effect of azithromycin on antibiotic resistance genes from pooled-template shotgun metagenomic screening. Counts per million total reads (CPM) of reads that matched to antibiotic resistance genes from the CARD database. Change in CPM (ΔCPM) between pooled samples before and after azithromycin or placebo, where red indicates higher in pooled-sample post azithromycin or placebo and blue indicates lower in pooled-sample post azithromycin or placebo. Resistance genes are grouped by function (as defined by CARD) where: red = aminoglycoside resistance genes, blue = beta-lactam resistance genes, green = efflux pump resistance genes, orange = fosfomycin resistance genes, purple = glycopeptide resistance genes, beige = macrolide resistance genes, pink = polymyxin resistance genes, light green = tetracycline resistance genes, and brown = other resistance genes. * indicates transmissible genes that were increased in the azithromycin group but not in the placebo group.

5.4.5 Macrolide resistance genes identified from sputum isolates

To investigate which species harboured transmissible resistance genes, bacteria were isolated from a subgroup of patients, before and after azithromycin (n=15, based on available sputum) and subjected to azithromycin susceptibility testing. The total proportion of azithromycin-resistant isolates significantly increased following azithromycin therapy ($p<0.0001$, Table 5.5), of which viridans streptococci were the only significant sub-group ($p=0.001$, Table 5.2). WGS identified macrolide resistance genes *erm*(B), *mel*, and *mef*(A), as well as *tet*(W) and *tet*(M) in resistant viridans streptococci (Table E4.1 of Appendix 4) Six of the seven streptococci with both *erm*(B) and *tet*(M) were found to contain both genes on a Tn916 mobile genetic element. Of the four azithromycin-resistant *H. parainfluenzae* isolated, two contained the putative mutation in the 50S ribosomal protein L4 (G65A) associated with macrolide resistance (Peric et al., 2003) while two had no recognised genetic mechanism for macrolide resistance (Table E4.1 of Appendix 4). Of the two azithromycin-resistant *S. aureus* isolates, one contained *erm*(A) and one contained *msr*(C) (Table E4.1 of Appendix 4).

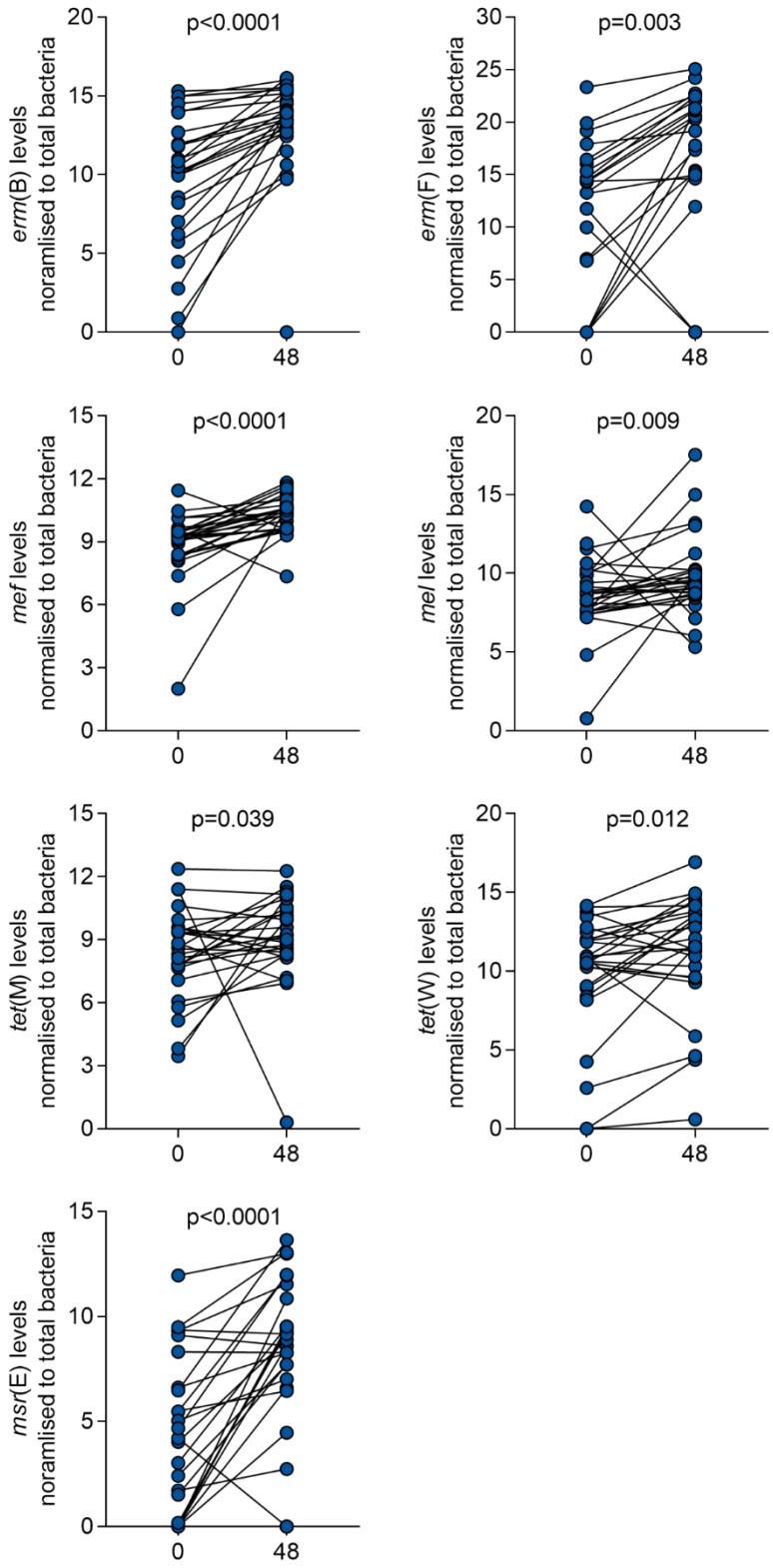

Figure 5.4: Effect of azithromycin on levels of screened antibiotic resistance genes. Levels of the seven antibiotic resistance genes before and after azithromycin. Genes were identified by pooled-template shotgun metagenomic sequencing quantified by qPCR and normalised to total bacteria.

Table 5.5: Proportion of azithromycin resistant isolates before and after therapy

	Before	**After**	**p-value**
Haemophilus influenzae	0/4 (0%)	0/0 (0%)	>0.99
Haemophilus parainfluenzae	2/13 (15%)	2/2 (100%)	0.057
Viridans streptococci	24/33 (73%)	33/33 (100%)	0.001
Staphylococcus aureus	1/2 (50%)	2/2 (100%)	>0.99
Total species	27/52 (52%)	37/37 (100%)	<0.0001

Fisher's exact test; Data available for 15 patients

5.5 Discussion

Long-term azithromycin treatment effectively manages exacerbations in adults with persistent uncontrolled asthma (Gibson et al., 2017), however there are concerns about the potential negative effects on the lower airway microbiology and antimicrobial resistance. The results here show that azithromycin has a selective effect on the lower airway microbiota however does not alter the total bacterial levels, nor does it increase the abundance of pathogenic bacteria. In fact, the amount of *H. influenzae* significantly decreased with azithromycin. However, concerning antimicrobial resistance carriage, azithromycin increased carriage of macrolide resistance and non-macrolide resistance genes. As these results were all determined using undirected methods, the results presented here represent a comprehensive investigation of the effect of azithromycin on the airway microbiome and resistome in asthma.

The multiple functions azithromycin elicits in chronic airway diseases make identifying the mechanism of action difficult. There was no significant decline in total bacterial load in patients receiving azithromycin, suggesting that broad antibiotic effects are minimal, however, the decline in phylogenetic diversity indicates a potentially selective effect of azithromycin. This is further evident in the weighted and unweighted UniFrac results, where unweighted UniFrac distance was significantly higher in azithromycin compared to placebo, however weighted UniFrac distance was not (p=0.74, data not shown). Unweighted UniFrac distance is calculated by the presence or absence of detected taxa whereas weighted UniFrac accounts for

relative abundance and is useful for examining differences in community structure (Lozupone et al., 2007). The discrepancy between weighted and unweighted UniFrac indicates azithromycin has specific selective effects that result in the loss of detection of certain taxa rather than a change in their relative abundance. The stark decline in *H. influenzae* load, but relatively unchanged *S. pneumoniae* load, following azithromycin exemplifies this. Results from LEfSe indicate that other members of the Gammaproteobacteria class are also affected by azithromycin.

The selective effects of azithromycin specific to *H. influenzae* (and potentially other Gammaproteobacteria) could reflect either direct effects (for example selective bacteriocidic properties) or indirect effects through non-antibiotic azithromycin properties. For example, azithromycin may improve immune-driven clearance of *H. influenzae*. Azithromycin has been previously shown to improve macrophage phagocytic function when given to patients with COPD (Hodge et al., 2008). Proper clearance of non-typeable *H. influenzae* from the lower airways is dependent on macrophage phagocytosis (Hodge et al., 2017; Ween et al., 2016). Therefore, the reduction in *H. influenzae* load following azithromycin reported here may be an indirect result from improved macrophage phagocytic ability.

Along with asthma, azithromycin has been shown to be an effective management option in a range of chronic respiratory diseases, including COPD (Albert et al., 2011), CF (Southern et al., 2012), and bronchiectasis (Altenburg et al., 2013; Wong et al., 2012). None of these studies reported significant changes to pathogen abundance. However, following long-term erythromycin in bronchiectasis a reduction in *H. influenzae*, and an increase in *P. aeruginosa* was reported (Rogers et al., 2014). No change to *P. aeruginosa* abundance (nor any other common respiratory pathogen measured) were observed, however *P. aeruginosa* is less common in asthma compared to bronchiectasis.

The other primary aim of this study was to assess the carriage of antibiotic resistance genes following azithromycin, as resistance is a known effect (Altenburg et al., 2013; Brusselle et al., 2013; Choo et al., 2018; Hare et al., 2015; Kastner and Guggenbichler, 2001; Malhotra-Kumar et al., 2007; Mustafa et al., 2017; Saiman et al., 2010; Samson et al., 2016; Serisier et al., 2013; Tramper-Stranders et al., 2007; Valery et al., 2013). After screening for all characterised antibiotic resistance genes, seven transmissible resistance genes were found that were higher in the airways post-azithromycin (compared to baseline) but not higher in placebo, of which five (*mel*, *msr*(E), *erm*(F), *mef*(A), and *erm*(B)) confer resistance to azithromycin. The increase in *tet*(W) and *tet*(M) are of interest as both are tetracycline resistance genes. From the streptococcal WGS data, *tet*(M) was found on the same mobile genetic element as *erm*(B), which a well characterised mobile genetic element (Clewell et al., 1995) and has been shown previously to increase in streptococci following macrolide therapy (Malhotra-Kumar et al., 2007). The observed increase in *tet*(W) appears to be novel in relation to macrolide therapy. *tet*(W) was detected in an azithromycin resistant *Streptococcus oralis* isolate however the further investigation is required to determine the extent of this and whether other prominent species carry this gene. These findings indicate the wider consequences of azithromycin therapy other than solely macrolide resistance and advocate for further research into the efficacy of non-antibiotic macrolides (Hodge et al., 2017).

This study has several limitations. While the pooled-template shotgun metagenomics analysis identified the top resistance genes increased across all included patients, other resistance genes that were not found at high abundance across multiple patients may have been missed. Further, the limited culture-based analysis failed to isolate any bacteria carrying *erm*(F) and *msr*(E), despite their increased carriage rate. It is likely that organisms other than streptococci, *Haemophilus* and *S. aureus* harbour these genes, which were not cultured in this study. For example, sequenced *Prevotella* and *Porphyromonas* isolates, which are common in

the airways, have regions that map to *erm*(F) whereas sequenced *Rothia* and *Pseudomonas* isolates have regions that map to *msr*(E). Inability to isolate relevant respiratory pathogens, such as *Moraxella catarrhalis* and *S. pneumoniae,* limit the ability to determine carriage of resistance genes in these organisms.

In summary, it was found that azithromycin did not affect the total amount of bacteria in the lower airways of patients with persistent uncontrolled asthma. However, azithromycin was associated with a significant decrease in the total diversity of taxa, including a significant decline in the pathogenic species *H. influenzae*. A significant increase in the carriage of antibiotic resistance genes was also found, which included macrolide-resistance genes and tetracycline-resistance genes, informing our understanding of the wider implications of long-term azithromycin therapy. This is the first study based on a randomized, double-blind, placebo-controlled trial to investigate the impact of azithromycin treatment on the asthma airway microbiome. The absence of selective increases in pathogenic bacteria with azithromycin treatment suggests that long-term therapy is not associated with deleterious impacts on airway microbiology. Indeed, significant changes in both airway microbiota diversity and levels of the important respiratory pathogen, *H. influenzae*, in those receiving azithromycin identifies potential contributors to treatment-associated clinical benefit.

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CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, I presented studies that have furthered our understanding of chronic airway diseases. With the lower airway environment of patients contributing to airflow limitation and respiratory insult, elucidating the complex ecology of the lower airways has opened new possibilities to characterise and target disease symptoms. Prior to this dissertation, it was known that the airways of patients were colonised by microbial communities and that the presence of microbes considered pathogenic could contribute to disease. It was further appreciated that the composition of the microbiota could also reflect aspects of disease and be potentially clinically informative, however it was poorly understood how the complex environment selected the microbiota and how this in-turn could affect disease symptoms.

The research of this dissertation aimed to characterise how the lower airway environment affected the composition of the resident microbiota and to assess the clinical value of these interactions in chronic airway disease. Specifically, the effect of three lower airway selective pressures on the microbiota composition were assessed: airway inflammation phenotype, inherent mucosal glycan variation, and long-term macrolides. The aims were effectively tested through the analysis of two randomised controlled trials cohorts. This dissertation has demonstrated that specific measurable aspects of the lower airway environment are associated with the microbiota and can be used to reflect underlying symptoms of disease, inform patient stratification, and predict disease course. Together, the studies of this dissertation provide a deeper understanding of the contributary roles of the lower airway physicochemical environment and microbiota to the underlying pathophysiology and clinical course of chronic airways disease. These findings can be further developed to tailor clinical care to the specific characteristics of a patient. The clinical insight gained from these studies and the future research now required off the back of these studies will now be discussed.

In Chapter 2, the selective role of the type of airway inflammation to the lower airway microbiota was investigated in asthma. It was found that patients with a neutrophilic inflammatory phenotype had an airway microbiota composition with a lower diversity and a high relative abundance of either *Moraxella* or *Haemophilus*, two taxa that are associated with opportunistic respiratory infections. By contract, the eosinophilic inflammatory phenotype was not associated with microbiota diversity or pro-inflammatory taxa. These results further our understanding of the neutrophilic asthma phenotype, which, compared to eosinophilic asthma, is less characterised and is associated with a less effective response rate to conventional therapies (Berry et al., 2007; Cowan et al., 2010). The microbiota composition in the airways of neutrophilic patients indicates these patients have a more selective respiratory environment where higher relative abundance of pathogenic taxa may contribute to inflammation.

The selective pressure of neutrophilic inflammation on airway microbiota diversity is well characterised in other chronic airway diseases, however is controversial in asthma. Sputum microbiota diversity of patients with COPD has been shown to inversely correlate with sputum neutrophilic markers (Dicker et al., 2018), with similar evidence in studies of CF (Zemanick et al., 2015) and bronchiectasis (Rogers et al., 2013). In asthma, eosinophilic inflammation (Durack et al., 2016; Huang et al., 2015; Simpson et al., 2016; Sverrild et al., 2017) has been previously associated with the microbiota composition, however, so too has neutrophilic inflammation (Green et al., 2014; Zhang et al., 2016), with the results of this dissertation supporting the latter.

The discrepancies between the effects of inflammation in asthma probably reflect the heterogeneity of the disease. To many, the diagnosis of "asthma" is an outdated term because it provides a restricted view of the heterogeneous mix of pathobiologically distinct mechanisms responsible for morbidity and mortality in patients (Pavord et al., 2018). Many subtypes of asthma have been defined (Wenzel, 2012), with some of these subtypes overlapping with other

diseases (Agusti et al., 2016; Pavord et al., 2018). The results presented in this dissertation support the stratification of patients with asthma by inflammatory phenotype, which has been previously found to be a clinically informative method (Berry et al., 2007; Cowan et al., 2010; Simpson et al., 2006). Large studies are now required to assess whether further stratification of patients by their airway microbiology is additive to clinical decision making. For example, are patients with neutrophilic asthma, and a reduced lower airway microbiota diversity less responsive to corticosteroids compared to neutrophilic patients with a higher microbiota diversity? Previous studies have proposed that the airway microbiota reduces corticosteroid efficacy through negating its anti-IL-8 effects in monocytes (Goleva et al., 2013), and in Chapter 2 I showed that microbiota diversity was associated with ICS dose.

In Chapter 3, I investigated the selective role of mucosal glycosylation to the lower airway microbiota. Demonstrated in bronchiectasis, I showed that a common genetic mutation (which limits the display of fucose on the mucosal surface) was a strong predictor of lower airway microbiology. This *secretor* mucosal genotype was also strongly associated with exacerbation frequency and lung function. In the context of previous studies in CF (Taylor-Cousar et al., 2009) and asthma (Innes et al., 2011; Kauffmann et al., 1996; Ronchetti et al., 2001), these results highlight the clinically important variable of mucosal glycosylation in patients with chronic airway diseases, which should be considered in the clinical setting.

Moving forward, there are several questions that now need to be addressed. As mucus glycosylation can influence multiple aspects of the lower airway environment (such as nutrient availability and adhesion sites for multiple microbes), what is/are the precise mechanism/s that allow secretor status to influence lower airway microbiology and disease severity in bronchiectasis? *P. aeruginosa* can bind to and metabolise many mucosal glycans, however the use of α(1,2)-fucosylated glycans has not been properly assessed. Studies assessing the role of mucus glycans on *P. aeruginosa* infection of the lower airways are now required, as well as

exploring alternative mucus glycan-related mechanisms of disease susceptibility. For instance, the influence of secretor status on viral infection susceptibility and the contribution of this to chronic airway diseases should now be investigated.

As described in Chapter 1.5, assessment of *FUT2* genotype has been found to influence susceptibility to a range of mucosal-associated infections and diseases. The mechanisms of action of this are likely to span across multiple facets of mucosal immunity, from facilitating pathogen infection, to microbiota-related mechanisms, such as modulation of immunity. It is important to know the degree to which mucosal glycosylation affects pathogen infection susceptibility and microbiota composition across the infection and disease spectrum. This will assess whether mucosal glycosylation has predictive capabilities in the clinical and public health settings. The increasing collection and analysis of large data repositories of populations provide an opportunity to explore effects of glycosylation variability on risks of infection and disease susceptibility at a population-level, as well as at an individual level, which should now be explored.

Apart from the predictive capabilities of secretor status, another potential area of translation is the development of novel therapies that target mucosal glycans and glycosylation. For instance, can therapies be developed to simulate the non-secretor glycan phenotype in secretor bronchiectasis patients? Inhalation of glycans that *P. aeruginosa* bind to has been shown to reduce *P. aeruginosa* load in the lungs of CF patients, most likely by acting as receptor decoys and limiting biofilm formation and epithelial adhesion (Boukerb et al., 2014; Hauber et al., 2008). Specifying the use of these types of therapies, based on a patient's mucosal glycosylation profile may improve efficacy.

Further, translation options exist for mucosal glycosylation that relate to the gut microbiota composition and susceptibility to allergic disease. A growing number of studies are

showing that early-life antibiotic exposure or an early-life dysregulated microbiota is linked with allergen sensitisation in later life (Zeissig and Blumberg, 2014). Potentially contributing to this is maternal secretor status, which has been shown to influence infant microbiota composition (Lewis et al., 2015; Rausch et al., 2017), likely through variable glycosylation of breast milk. As non-secretors are more susceptible to asthma (Kauffmann et al., 1996), psoriasis (Tang et al., 2014), and early life eczema (Sprenger et al., 2017), the relationship between glycan fucosylation and the microbiome composition may contribute to the development of allergic conditions. This now requires further investigation and could lead to novel therapeutic strategies. For example, might breast milk, supplemented with α(1,2)-fucose reduce allergic disease susceptibility in at risk infants? Oral supplementation with α(1,2)-fucose has been shown to be well tolerated and shifts the intestinal microbiota in healthy adults (Elison et al., 2016).

In Chapter 4 and Chapter 5, I investigated the effect of macrolides on the lower airway microbiota. This was tested first by measuring the effect of macrolides on bacterial resistance gene carriage and second by measuring the effect of macrolides on the microbiota composition and pathogen prevalence. In assessing the carriage of resistance genes, a novel metagenomic approach was developed, which can improve the monitoring of antibiotic resistance globally. This method identifies how all antibiotic resistance genes change within a population, in a cost-effective manner. In the AMAZES cohort, this method was applied to show that macrolide and tetracycline resistance genes increased following azithromycin. Future research is now required to determine whether this method can be applied to other populations with a high antibiotic burden. For example, in aged care facilities (Lim et al., 2015), agricultural practices (Thanner et al., 2016), and countries with poor antibiotic stewardship (Cox et al., 2017), antimicrobial resistance is of particular concern, however the development and transmission of resistance mechanisms in these populations is poorly understood (World Health Organisation,

2018). Further validation of this pooled-template shotgun metagenomic method is now required in these populations where monitoring of antibiotic resistance is warranted. Application of such a method can improve the early detection of emerging novel resistance mechanisms that would otherwise spread unnoticed.

The results of Chapters 4 and 5 also provide important insight into the effects of macrolides in chronic airway disease. Currently, the use of macrolides as a maintenance therapy for patients with chronic airway diseases is cautioned with either the potential increase in carriage of macrolide resistant organisms (Chalmers et al., 2017; Yang et al., 2017), or the possible lower airway infection by *P. aeruginosa* (Chalmers et al., 2017). The results of this dissertation support the former caution, but do not support the latter; no increases in the relative abundance of pathogenic organisms were found in sputum. In fact, assessment of the lower airway microbiota and pathogen abundance in asthma identified a strong reduction in *H. influenzae* following azithromycin. However, the increased carriage of resistance can increase the pathogenicity of organisms. For example, macrolide-resistant *S. aureus,* which increased following azithromycin use in a trial of children with bronchiectasis or chronic suppurative disease (Valery et al., 2013), can represent a clinically important pathogen.

It remains to be known whether the reduction in *H. influenzae* was due a direct antibiotic effect of macrolides or an indirect effect through other modulatory functions of macrolides. The body of literature that shows that macrolides can restore macrophage phagocytic function (Hodge et al., 2008) and improve *H. influenzae* lower airway clearance (Hodge et al., 2017; Ween et al., 2016), credit an indirect effect of macrolides. Future studies are now required to validate this non-antibiotic effect of macrolides in asthma, such as testing the clinical efficacy of non-antibiotic macrolides (Hodge et al., 2017) at reducing exacerbations. Non-antibiotic macrolides exist however have their effect at reducing exacerbations in patients with persistent, severe asthma has not been tested. Indeed, if non-antibiotic macrolides are effective at reducing

exacerbations and are adopted into clinical practice, then the concern of antibiotic resistance from azithromycin, as reported in Chapter 5, can be offset.

Another clinically important question related to Chapter 5 is: does the reduction in *H. influenzae* from macrolides also reduce patient's frequency of exacerbation? *H. influenzae* in the lower airways is associated with airway inflammation (Green et al., 2014; Huang et al., 2015) and exacerbations (Wang et al., 2017), therefore may contribute to exacerbations in the AMAZES cohort. Studies are now required to determine whether patients with high levels of *H. influenzae* respond better to long-term macrolide therapy, which would be a major step forward in predicting macrolide treatment efficacy.

In summary, the results presented in this dissertation contribute to our understanding of the complex interactions in the lower airways of patients with chronic airway disease. They identify the effects of inflammation, mucus composition, and pharmacological treatments on the airway microbiota as well as form the foundation for future research. Clinically, these studies also provide important insight that contributes to the ongoing effort to personalise disease symptoms in patients to predict disease trajectory and selection of effective treatments.

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APPENDICES

Appendix 1: Supplementary Material from Chapter 2

The contents of this supplement have been published as part of:

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Supplementary Methods

Exclusion criteria

Asthma diagnosis was established using American Thoracic Society guidelines based on current episodic respiratory symptoms, clinical diagnosis and evidence of variable airflow obstruction (1987). Participants with asthma were included if stable but symptomatic, despite being prescribed maintenance inhaled corticosteroid (ICS) and long acting bronchodilator treatment with an Asthma Control Questionnaire 6 (ACQ6) score >0.75 (Juniper et al., 1999).

Participants with an FEV_1 <40% predicted, current smokers, ex-smokers who had ceased smoking in the previous year and those with a recent (past four weeks) exacerbation or respiratory infection were excluded. Patients were also excluded if they had received antibiotic treatment in the preceding month. Those with significant smoking related air-space disease (ex-smokers >10 pack year history and DLCO/VA <70% predicted OR smoking history >10 pack years and exhaled carbon monoxide >10 parts per million) were also excluded. This study was conducted in accordance with the amended Declaration of Helsinki. Local institutional review boards approved the protocol and written informed consent was obtained from all participants.

Institutional centres

Sputum samples were collected from eight Australian centres:

1. Hunter Medical Research Institute, Newcastle NSW Australia
2. The Prince Charles Hospital, Chermside, QLD, Australia
3. Princess Alexandra Hospital, Woolloongabba QLD, Australia
4. Royal Adelaide Hospital, Adelaide SA, Australia
5. Sir Charles Gairdner Hospital, Nedlands WA, Australia
6. Woolcock Institute of Medical Research, Glebe NSW, Australia
7. Concord Repatriation General Hospital, Concord NSW, Australia
8. Liverpool Hospital, Liverpool NSW, Australia

Patient inflammatory phenotyping

Patient sputum was dispersed using dithiothreitol and inflammatory cells were counted as a percentage of total sputum cells. Inflammatory subtype was determined as described below. Neutrophilic cut-off values were age-dependent as described previously (Brooks et al., 2013; Simpson et al., 2014).

Neutrophilic phenotype

Neutrophil% (<20 years old)	≥75.57%
Neutrophil% (20-40 years old)	≥61.61%
Neutrophil% (40-60 years old)	≥63.25%
Neutrophil% (>60 years old)	≥67.25%

Eosinophilic phenotype

Eosinophil%	≥3%

Paucigranulocytic phenotype

Eosinophil%	≤3%

Neutrophil% (<20 years old) ≤75.57%

Neutrophil% (20-40 years old) ≤61.61%

Neutrophil% (40-60 years old) ≤63.25%

Neutrophil% (>60 years old) ≤67.25%

Mixed granulocytic phenotype

Eosinophil% ≥3%

Neutrophil% (<20 years old) ≥75.57%

Neutrophil% (20-40 years old) ≥61.61%

Neutrophil% (40-60 years old) ≥63.25%

Neutrophil% (>60 years old) ≥67.25%

Table E1.1: 16S rRNA sequencing information

Median read count (IQR)	12792 (8060, 16595)
Subsample depth	1732
Samples excluded	7
Median Good's coverage (IQR)	0.952 (0.942, 0.963)

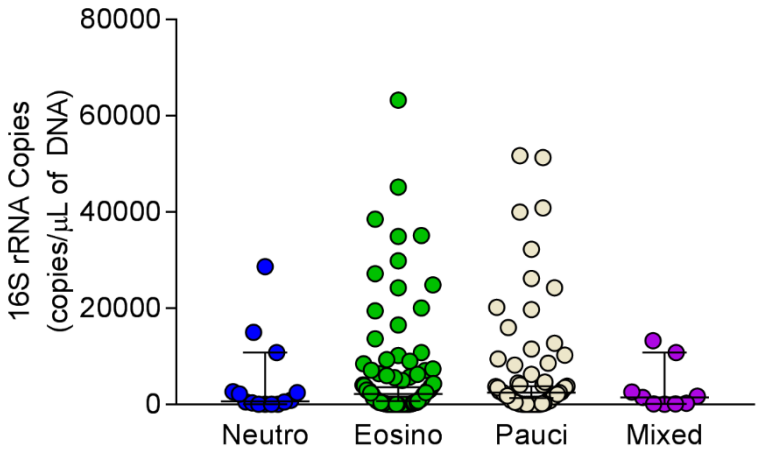

Figure E1.1: Bacterial burden as assessed by 16S rRNA gene copy number. Bars show the median±95% CI. Statistical significance was assessed by Kruskal-Wallis one-way ANOVA with Dunn's post hoc test. No significant difference between phenotypes.

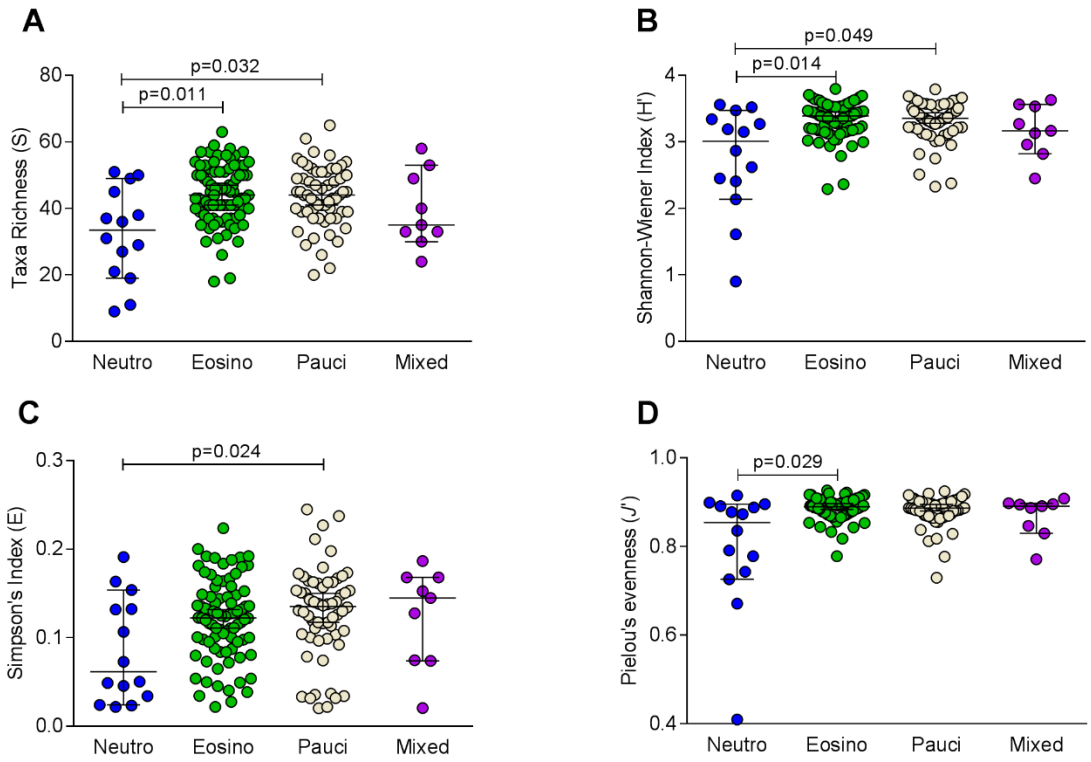

Figure E1.2: Alpha diversity measures among asthma phenotypes. A) Taxa richness, B) Shannon-Wiener index, C) Simpson's evenness index, D) Pielou's evenness. Bars show the median±95% CI. Statistical significance was assessed by Kruskal-Wallis one-way ANOVA with Dunn's post hoc test.

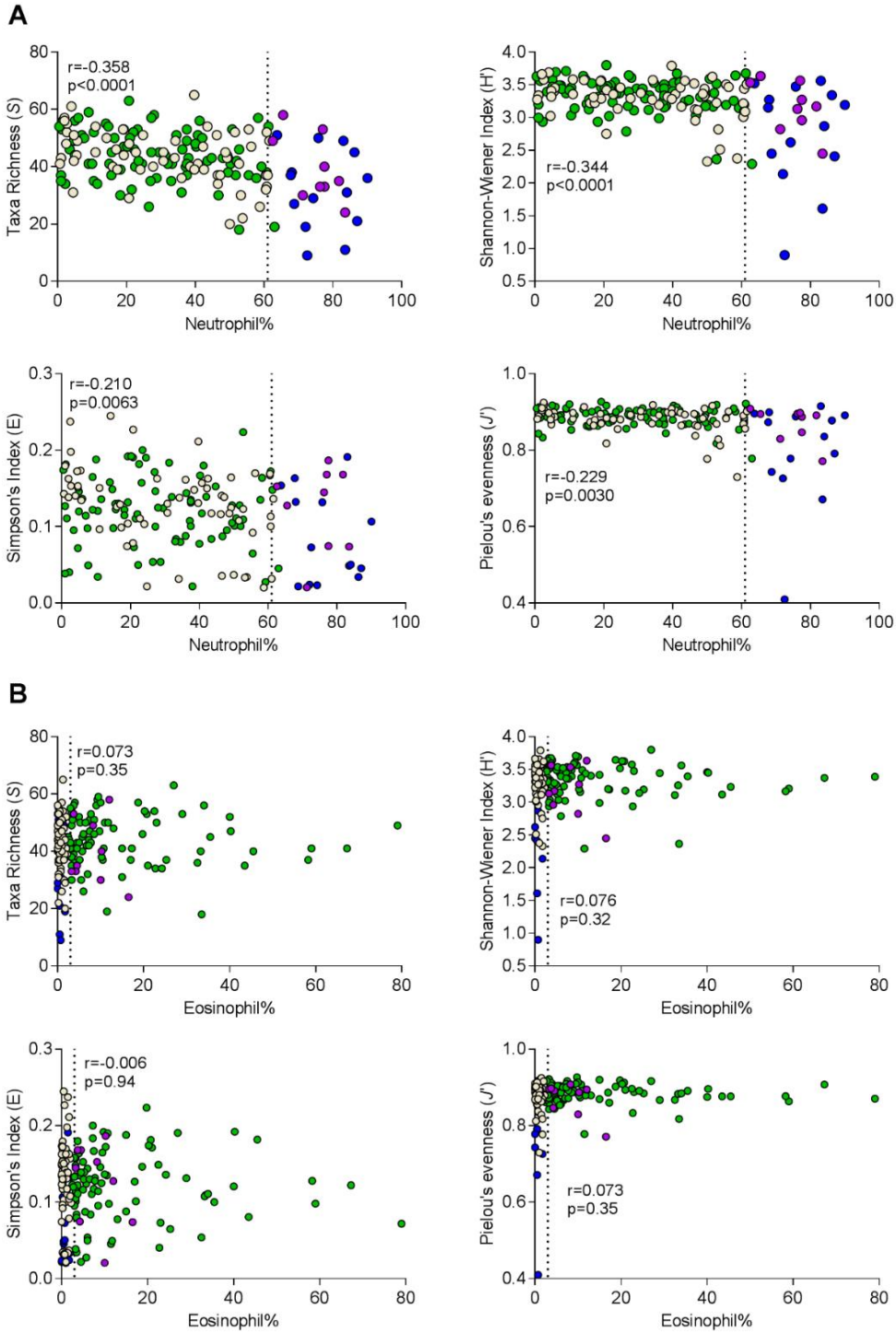

Figure E1.3: Correlations between sputum neutrophil/eosinophil counts (as a percentage of total cell count) and alpha diversity measures. A) Neutrophil % where dotted line at 61% neutrophils indicates phenotype cut-off point. B) Eosinophil % where dotted line at 3% eosinophils indicates phenotype cut-off point. Colours represent asthma phenotype, based on neutrophilia or eosinophilia where blue= >61% neutrophils, green= >3% eosinophils, yellow= <61% neutrophils and <3% eosinophils (paucigranulocytic), and purple= both >61% neutrophils and >3% eosinophils (mixed). Statistical significance was assessed by Spearman's rank correlation.

Table E1.2: PERMANOVA analysis testing significance of variance of Weighted UniFrac and Bray-Curtis Distance of sputum microbiota between asthma phenotypes (permutations = 9999)

Matrix	Source	Df	SS	MS	Pseudo-F	P(perm)
Weighted UniFrac	Phenotype	3	9444.4	3148.1	3.9969	0.0004
	Residual	163	128390	787.64		
	Total	166	137830			
Bray-Curtis	Phenotype	3	8215.4	2738.5	3.3694	0.0001
	Residual	163	132480	812.74		
	Total	166	140690			

Table E1.3: Pairwise PERMANOVA analysis testing significance of variance of Weighted UniFrac and Bray-Curtis Distance of sputum microbiota between asthma phenotypes (permutations = 9999)

Matrix	Groups	T	P(perm)	Unique perms
Weighted UniFrac	Pauci vs Neutro	2.52	<0.01	9943
	Pauci vs Eosino	1.28	0.13	9928
	Pauci vs Mixed	0.80	0.62	9952
	Neutro vs Eosino	3.30	<0.0001	9924
	Neutro vs Mixed	1.20	0.21	9876
	Eosino vs Mixed	1.19	0.19	9939
Bray-Curtis	Neutro vs Eosino	2.89	<0.0001	9928
	Neutro vs Pauci	2.43	<0.001	9918
	Neutro vs Mixed	1.18	0.19	9888
	Eosino vs Pauci	1.15	0.17	9921
	Eosino vs Mixed	0.96	0.50	9899
	Pauci vs Mixed	0.78	0.83	9922

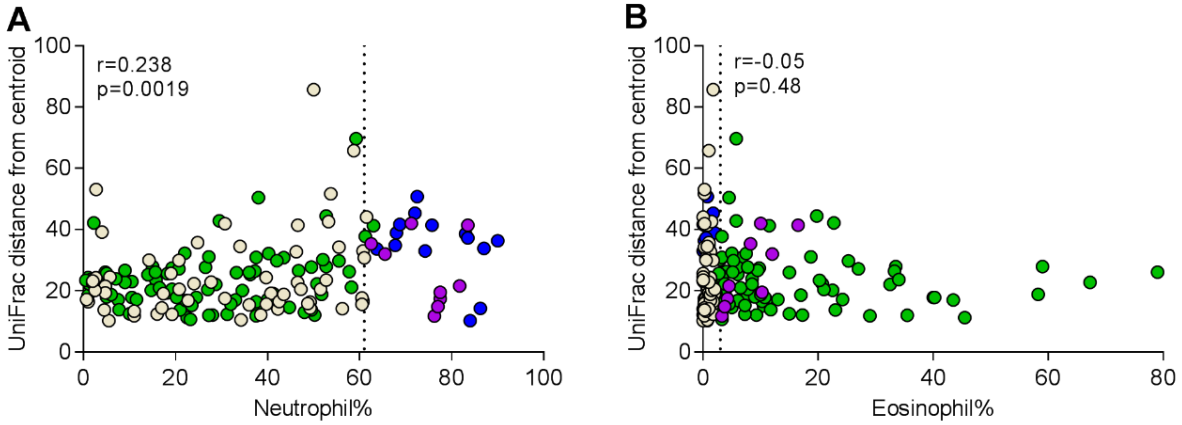

Figure E1.4: Correlations between sputum neutrophil/eosinophil counts (as a percentage of total cell count) and weighted UniFrac distance from centroid. A) Neutrophil % where dotted line at 61% neutrophils indicates phenotype cut-off point. B) Eosinophil % where dotted line at 3% eosinophils indicates phenotype cut-off point. Colours represent asthma phenotype, based on neutrophilia or eosinophilia where blue= >61% neutrophils, green= >3% eosinophils, yellow= <61% neutrophils and <3% eosinophils (paucigranulocytic), and purple= both >61% neutrophils and >3% eosinophils (mixed). Statistical significance was assessed by Spearman's rank correlation.

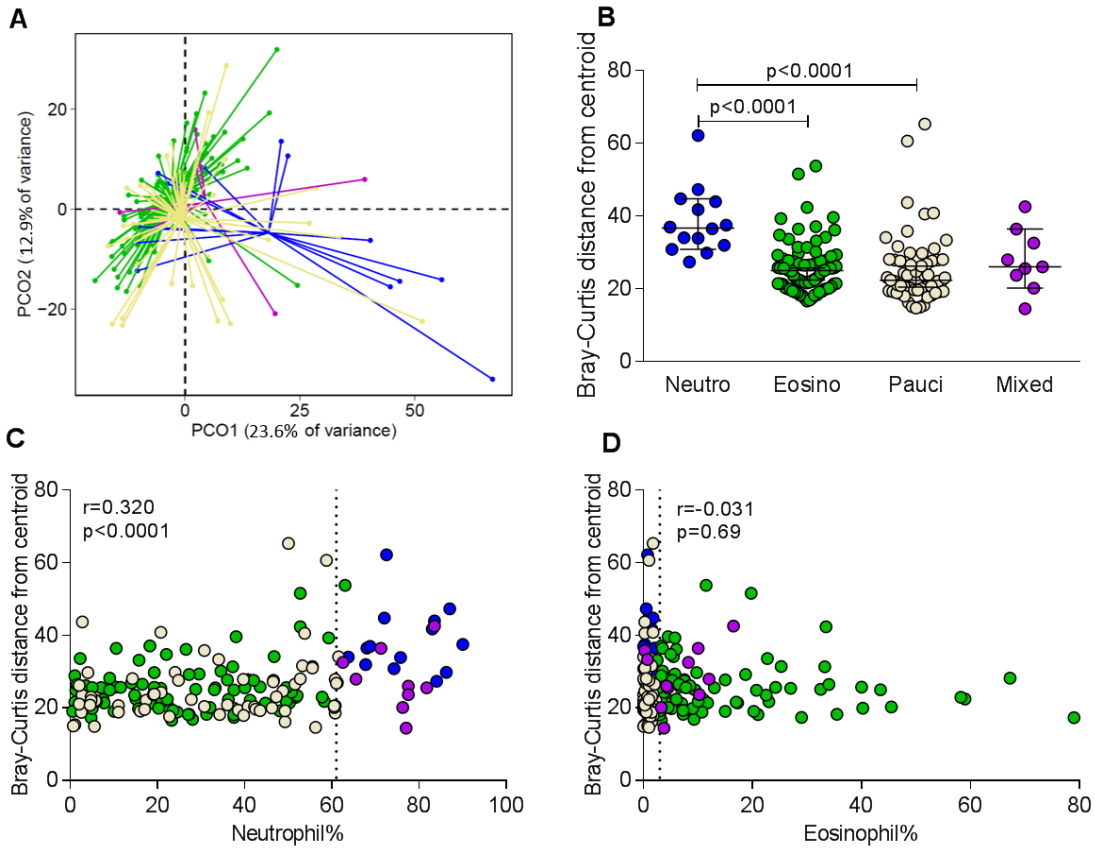

Figure E1.5: A) Principal Coordinate Analysis (PCoA) of asthma phenotype groups based on Bray-Curtis similarity distances. The first two principal coordinates are plotted on the *x*- and *y*-axes, respectively (representing 36.5% of the total variation). B) Microbiota dispersion grouped by asthma phenotype. Distance from centroid calculated from Bray-Curtis dissimilarity matrix. C,D) Correlations between sputum inflammatory cell percentages and distance from centroid. C) Sputum neutrophil % vs Bray-Curtis distance from centroid. D) Sputum eosinophil % vs Bray-Curtis distance from centroid.

Table E1.4: Similarity of percentages (SIMPER) analysis comparing taxa relative abundances between neutrophilic and eosinophilic phenotype groups. Showing thirteen top contributing taxa which collectively account for approximately 50% of variance between groups.

	Neutrophilic	Eosinophilic		
Species	Av. Abund	Av. Abund	Av. Diss	Contrib %
Haemophilus	0.41	0.2	4.49	8.89
Prevotella	0.23	0.33	2.61	5.16
Streptococcus II	0.13	0.27	2.58	5.11
Streptococcus I	0.45	0.51	2.34	4.63
Veillonella	0.2	0.22	1.98	3.91
Moraxella	0.13	0	1.88	3.72
Neisseria	0.11	0.19	1.65	3.26
Rothia	0.1	0.19	1.52	3
Actinomyces sp. uncultured bacterium	0.1	0.16	1.5	2.96
Gemella	0.09	0.17	1.26	2.49
Leptotrichia	0.08	0.11	1.2	2.38
Actinomyces sp. oral clone DR002	0.05	0.08	1.1	2.17
Porphyromonas	0.04	0.11	1.1	2.17

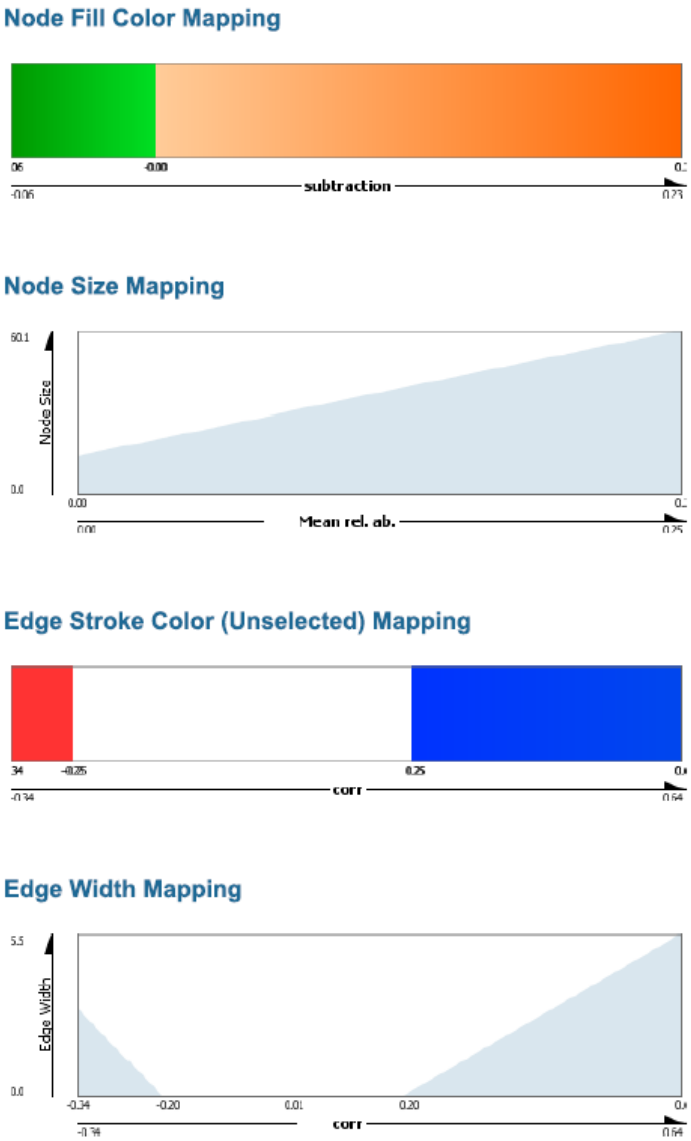

Figure E1.6: Figure legend for bacterial network analysis. Showing weight and colour assigned to edges and nodes.

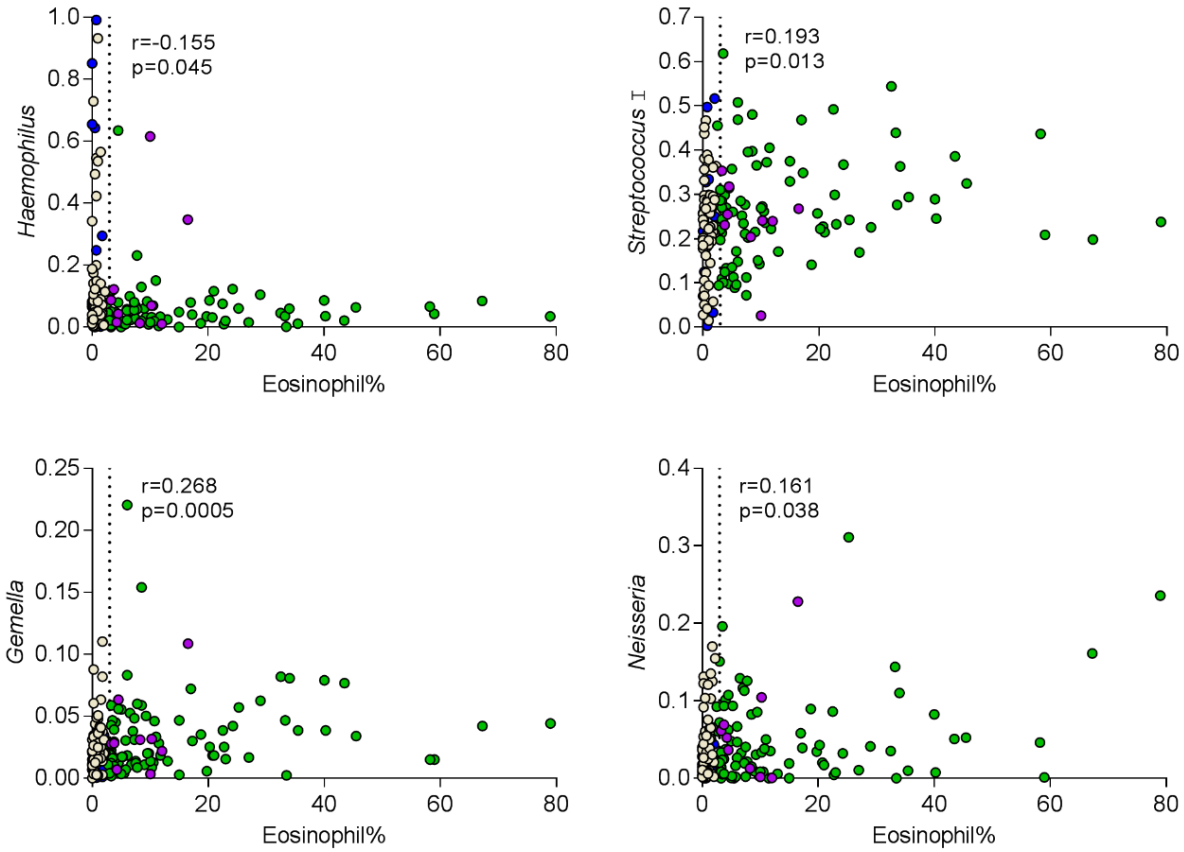

Figure E1.7: Taxa which significantly correlated with eosinophil %. Colours represent asthma phenotype, based on neutrophilia or eosinophilia where blue= >61% neutrophils, green= >3% eosinophils, yellow= <61% neutrophils and <3% eosinophils (paucigranulocytic), and purple= both >61% neutrophils and >3% eosinophils (mixed). Dotted line at 3% eosinophils indicate phenotype cut-off points. Statistical significance was assessed by Spearman's rank correlation.

Table E1.5: Top: PERMANOVA analysis, Bottom: Pairwise PERMANOVA on non-dominant microbiome. Bray-Curtis Distance of sputum microbiota on the genera level, grouped by asthma phenotype (permutations = 9999).

Source	**Df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**
Phenotype	3	5381	1793.7	2.378	0.0004
Residual	163	122950	754.3		
Total	166	128330			

Groups	**T**	**P(perm)**	**Unique perms**
Neutro vs Eosino	2.31	<0.0001	9922
Neutro vs Pauci	2.26	<0.001	9911
Neutro vs Mixed	1.11	0.24	9857
Eosino vs Pauci	0.91	0.65	9904
Eosino vs Mixed	0.79	0.86	9921
Pauci vs Mixed	0.73	0.92	9924

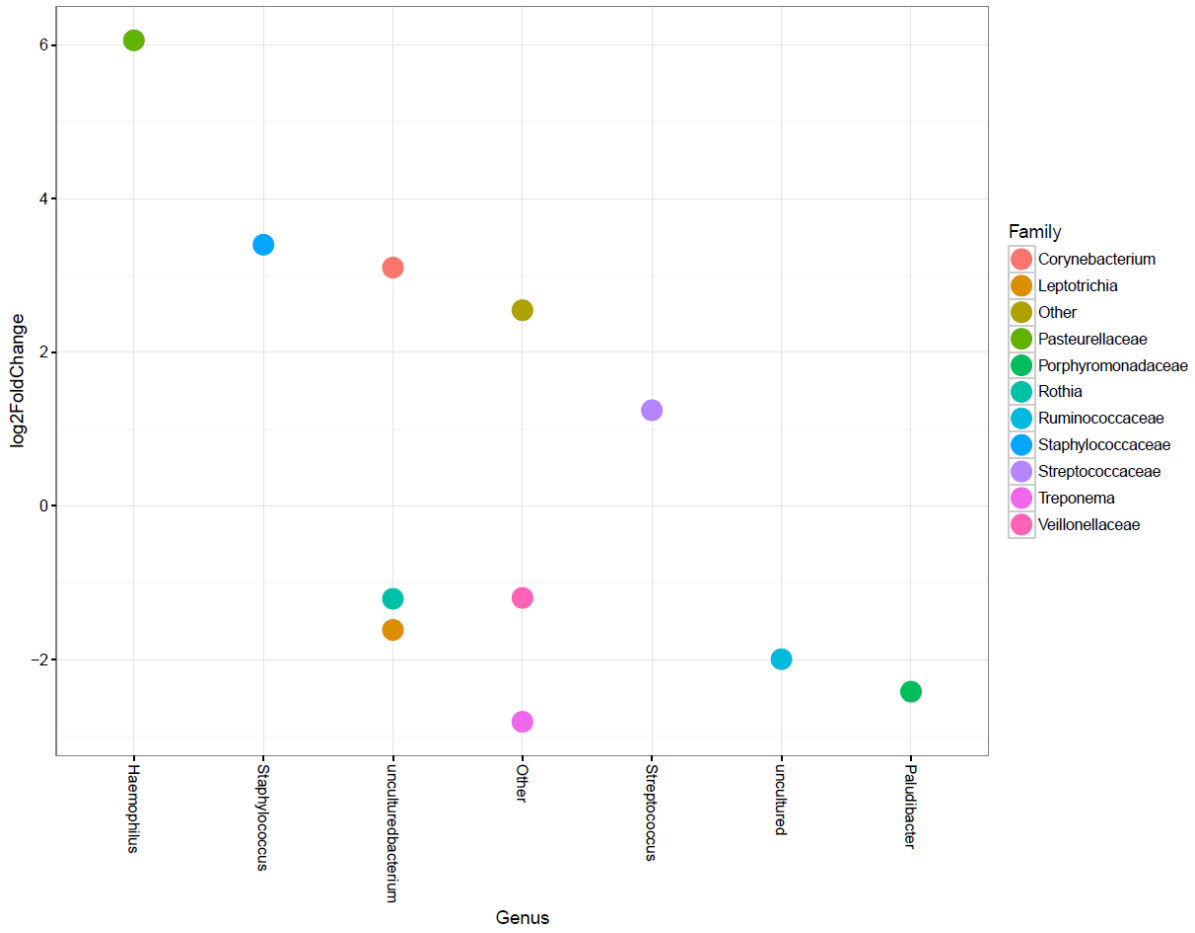

Figure E1.8: Normalised, $\log_2$ fold changes of non-rarefied taxa read counts which significantly ($p<0.05$) differed between neutrophilic and eosinophilic phenotypes. Positive fold change indicates taxon which were significantly higher counts in neutrophilic participants, negative fold change indicates taxon which were significantly higher in eosinophilic participants. Showing that, when *Haemophilus* or *Moraxella* dominance do not influence data (due to non-rarefied count data as opposed to relative abundance), multiple taxa remain significantly different between neutrophilic and eosinophilic participants.

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Simpson, J.L., Phipps, S., Baines, K.J., Oreo, K.M., Gunawardhana, L., and Gibson, P.G. (2014). Elevated expression of the NLRP3 inflammasome in neutrophilic asthma. The European respiratory journal *43*, 1067-1076.

Appendix 2: Supplementary Material from Chapter 3

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Supplementary methodology

Bronchiectasis subject inclusion criteria

1. Able to provide written informed consent.
2. Confirmed diagnosis of bronchiectasis by high resolution computed tomography within 3 years.
3. Airways obstruction on spirometry (ratio FEV_1/ FVC <0.7) and FEV_1 ≥25% predicted.
4. Chronic productive cough with at least 5 mLs sputum production per day.
5. At least two exacerbations of bronchiectasis requiring either oral or intravenous supplemental antibiotic therapy (of at least 7 days duration on each occasion) in the prior 12 months.
6. Aged 20-85 inclusive.
7. Clinically stable for at least four weeks (defined as no symptoms of exacerbation, no requirement for supplemental antibiotic therapy, and FEV_1 within 10% of best recently recorded value where available).

Exclusion criteria

1. Bronchiectasis as a result of CF or focal endobronchial obstruction.
2. Currently active tuberculosis or non-tuberculous mycobacterial (NTM) infection. Subjects with evidence of prior pulmonary NTM infection could be included only if

they have completed a course of therapy that is deemed successful on the basis of negative NTM cultures following cessation of therapy. All subjects required a negative NTM culture prior to screening.

3. Any symptoms or signs to suggest recent deterioration in respiratory disease, including exacerbation of pulmonary disease (as previously defined) in the preceding 4 weeks.
4. Any change to medications in the preceding 4 weeks.
5. Prescription of either oral or intravenous antibiotic therapy in the preceding 4 weeks.
6. Cigarette smoking within the preceding 6 months.
7. Any history of malignant arrhythmia (unless in the immediate post-myocardial infarction period and not requiring any regular therapy) or QTc prolongation on baseline electrocardiogram.
8. Any of the following within the three (3) months prior to enrolment:
    a. Acute myocardial infarction
    b. Acute cerebrovascular accident
    c. Major surgery
9. History of any of the following:
    d. Active malignancy (excepting non-melanoma skin malignancies that have been treated and considered cured)
    e. Listed for transplantation
    f. Any other significant active illness likely to affect the patient's survival within 12 months
    g. Receiving long-term domiciliary oxygen therapy
10. Allergy to macrolide antibiotics, other than minor, dose-related gastrointestinal intolerance that would not be anticipated to recur with low-dose erythromycin.

11. Any prescription or receipt of long-term macrolide antibiotics, or receipt of a treatment course within 4 weeks.
12. Predominant diagnosis of emphysema (rather than bronchiectasis) on high-resolution computed tomography scan of the chest.
13. Requirement for supplemental oxygen therapy.
14. Inability to complete required study procedures for whatever reason (including 6-minute walk test, hypertonic saline sputum induction).
15. Respiratory symptoms (including cough, sputum production, recurrent exacerbations) not predominantly the result of bronchiectasis in the opinion of the principal investigator; where treatable causes for exacerbations existed, these were treated before considering trial enrolment.

Table E2.1: Summary of relative abundance of the dominant infective microbe in sputum of bronchiectasis patients

Genus	Number of patients	% Average relative abundance (SD)
Haemophilus	33	85.6 (18)
Pseudomonas	25	87.0 (14)
Veillonella	9	33.6 (8)
Prevotella	9	40.3 (13)
Streptococcus	4	44.2 (21)
Pasteurella	2	93.0 (9)
Stenotrophomonas	1	80.8
Staphylococcus	1	25.6
Porphyromonas	1	72.6
Neisseria	1	18.0
Moraxella	1	79.1
Leptotrichia	1	34.6
Flavobacterium	1	16.7
Burkholderia	1	58.1
Bordetella	1	54.2
Achromobacter	1	52.7
Abiotrophia	1	92.7

Table E2.2: Fungal Supplementary data. Samples were each assigned as either positive or negative for *C. albicans* or *A. fumigatus* based on qPCR CT score, referenced to culture positive standard curves. Details of each standard curve and detection and cut-off thresholds are detailed below. Approximate copy numbers for standards were calculated based on DNA concentration of positive control (measured by Qubit fluorometer), fungal genome size, and number of gene copies per genome. Samples were run in triplicate and averaged.

C. albicans isolate standard			
Sample	Copy number	Average Cycle Threshold	std
Dil1:10^0[a]	621600		
Dil1:10^1	62160	22.901	0.118
Dil1:10^2	6216	27.790	0.288
Dil1:10^3	621.6	31.247	0.165
Dil1:10^4	62.16	34.211	0.737
Dil1:10^5	6.216	37.231	
% amplification efficiency		92.77%	
R^2		0.9879	

Detection limit of 35 CT (or 36.4 copies) assigned to *C. albicans* qPCR based on standard curve

[a] 1:10^0 dilution failed to produce sufficient CT, likely due to interference with PCR inhibitors or buffer concentration imbalance. No samples produced a CT <27 therefore this dilution was ignored.

A. fumigatus isolate standard			
Sample	Copy number	Average Cycle Threshold	std
Dil1:10^0	3763200	13.022	0.009
Dil1:10^1	376320	16.392	0.003
Dil1:10^2	37632	19.916	0.024
Dil1:10^3	3763.2	23.392	0.107
Dil1:10^4	376.32	27.237	0.153
Dil1:10^5	37.632	30.927	0.500
% amplification efficiency		90.02%	
R^2		0.9995	

Detection limit of 34 CT (or 4.76 copies) assigned to *A. fumigatus* qPCR based on standard curve

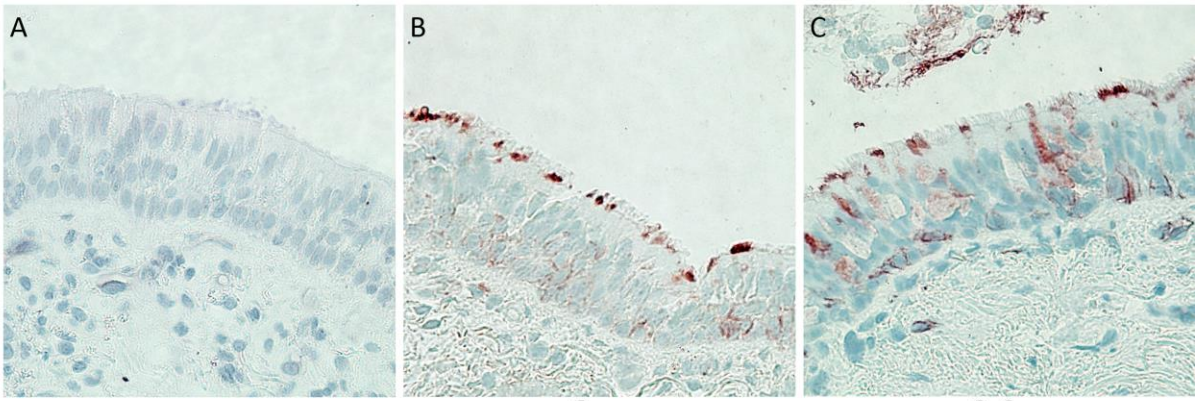

Figure E2.1: Endobronchial biopsy stain by *FUT2* genotype. UEA-1 lectin staining, which is specific for α(1,2)-fucosylated Lewis glycans, of bronchial biopsies from bronchiectasis patients with *sese* (A), *Sese* (B), and *SeSe* (C) genotypes. Staining is located on apical cell surfaces and within secretory vesicles of *Sese* and *SeSe,* but absent in *sese* biopsies. There was no clear difference in staining intensity between biopsies from *Sese* and *SeSe* patients.

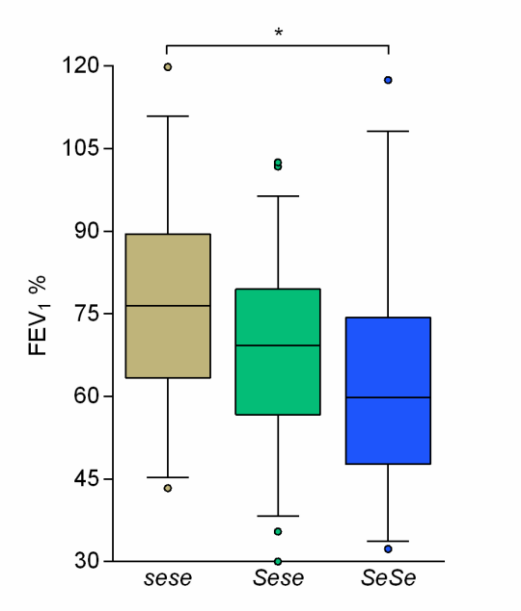

Figure E2.2: Effect of *FUT2* genotype on FEV_1 %. Box and whisker plots show median, IQR, and 5th and 95th percentiles (dots show outliers) of pre-bronchodilator FEV_1 as a percentage of the predicted value. *p=0.023 by Tukey's post-hoc test.

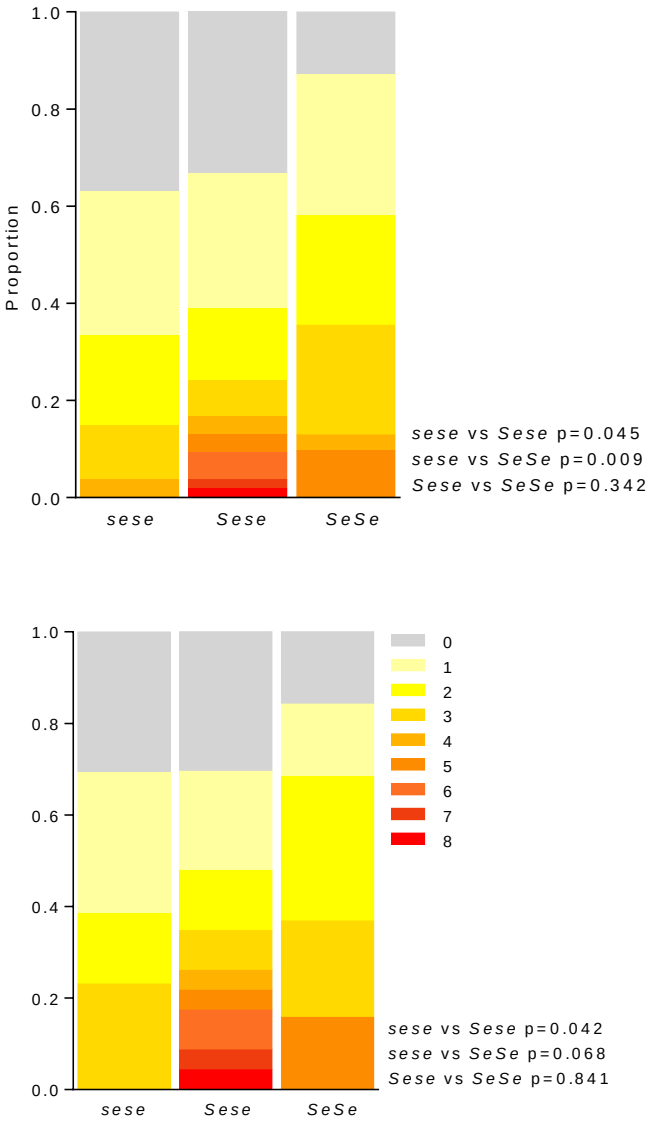

Figure E2.3: Effect of *FUT2* genotype on physician defined pulmonary exacerbation count. Proportion of patients who had a physician defined pulmonary exacerbations (PDPE) over the 48 weeks of the trial. Colours indicate number of PDPEs. Top –All patients, Bottom –Patients in the placebo group only. P values calculated by Wald test.

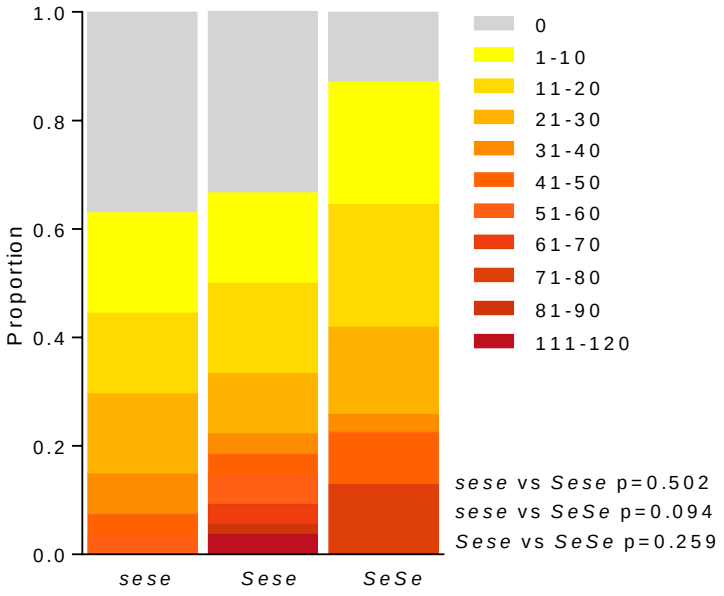

Figure E2.4: Effect of *FUT2* genotype on time on antibiotics. Number of days on antibiotics due to pulmonary exacerbation were recorded over the course of trial. Showing total patients (receiving either placebo or erythromycin). Time on antibiotics in 10-day groups, for purpose of graphing. P values calculated by Wilcoxon rank-sum test based on ungrouped data.

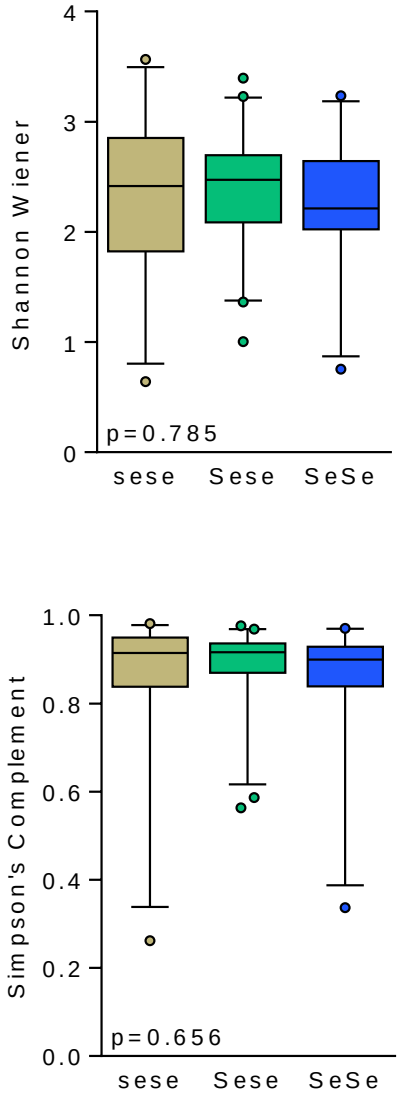

Figure E2.5: Effect of *FUT2* genotype on non-dominant taxa alpha diversity. Shannon Weiner Index and Simpson's Complement Index of rescaled relative abundance (excluding *Pseudomonas aeruginosa* and *Haemophilus influenzae*, when dominant). P values calculated by ANOVA (top) and Kruskal-Wallis (bottom). This indicates no significant difference of within patient microbiome diversity of evenness between secretor genotypes.

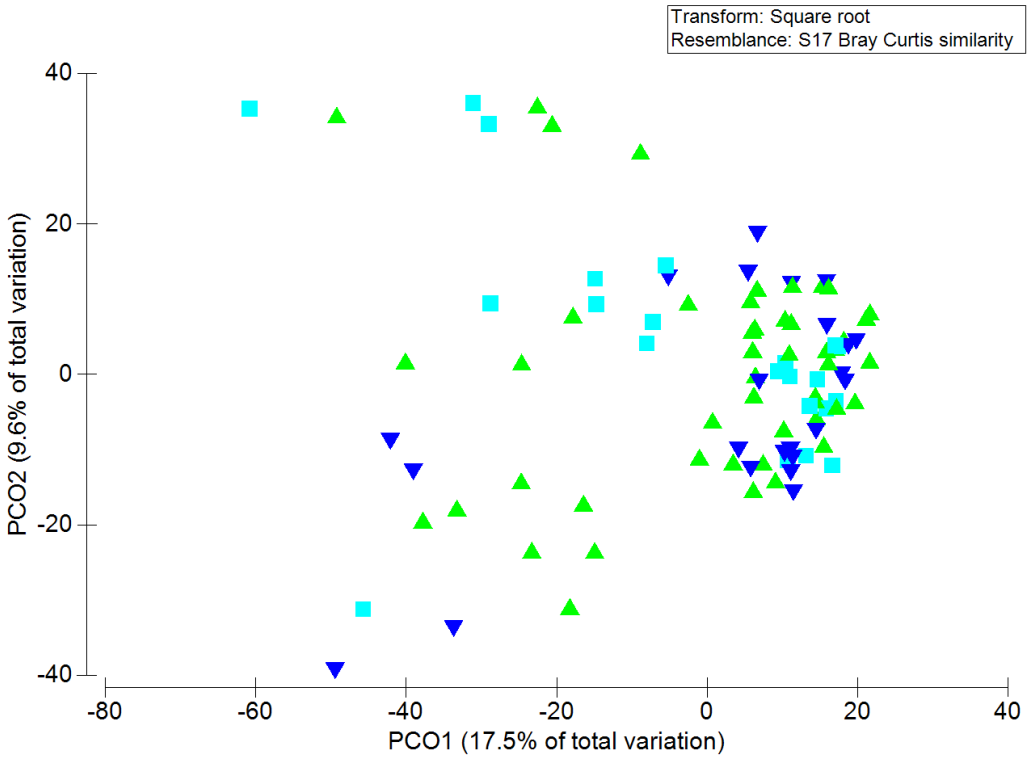

Figure E2.6: Effect of *FUT2* genotype on Bray-Curtis principal coordinate (PCO) analysis. PCO1 and PCO2 plot of Bray-Curtis similarity of rescaled relative abundance (excluding *Pseudomonas aeruginosa* and *Haemophilus influenzae*, when dominant) between *sese* (dark blue), *Sese* (green), and *SeSe* (light blue) patients. This indicates that the microbiota of patients, excluding *P. aeruginosa* and *H. influenzae*, when dominant, is not different. PERMANOVA p=0.777.

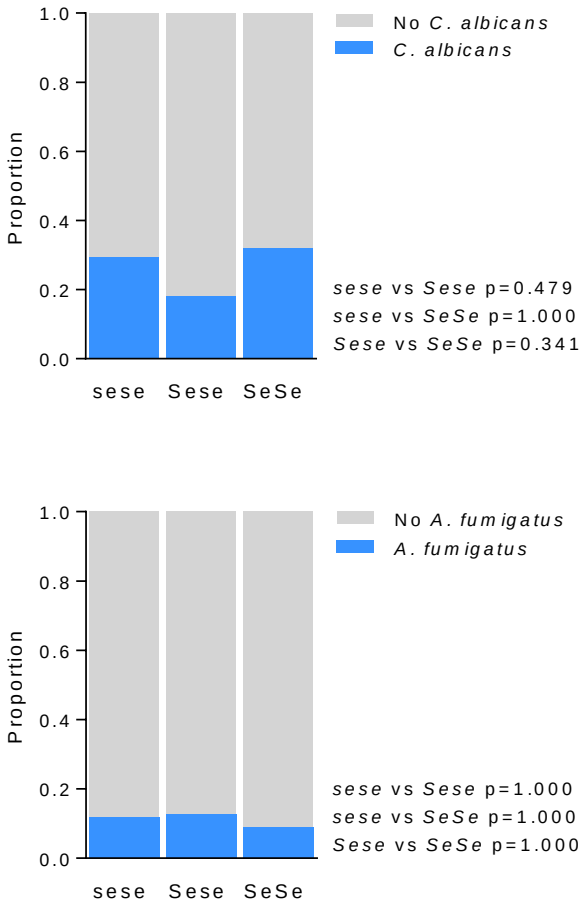

Figure E2.7: Effect of *FUT2* genotype on airway fungal predominance. Detection of *Candida albicans* (top) and *Aspergillus fumigatus* (bottom). P values calculated by Fishers exact test.

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Appendix 3: Supplementary Material from Chapter 4

The contents of this supplement have been published as part of:

> **Taylor, S.L.**, Leong, L.E.X., Mobegi, F., Choo, J.M., Burr, L.D., Wesselingh, S., and Rogers, G.B. (2018) Understanding the impact of antibiotic therapies on the respiratory tract resistome: a novel pooled-template metagenomic sequencing strategy. Multidisciplinary Respiratory Medicine *13*, 9-14.

Table E3.1: Characteristics of patients at baseline

	Erythromycin (n=32)	Placebo (n=31)
Age, mean yrs (std)	63.5 (7.9)	64.1 (9.9)
Gender, female (%)	19 (59.4)	15 (48.4)
Duration of bronchiectasis, mean yrs (std)	45.3 (20.2)	41.4 (23.3)
Ex-smoker, n (%)	8 (25)	7 (22.6)
Inhaled corticosteroids, n (%)	5 (15.6)	4 (12.9)
PDPE, median (IQR)	1 (0-2)	2 (1-3)
FEV_1/FVC, mean (std)	0.676 (0.09)	0.701 (0.09)
FEV_1 absolute, mean (std)	1.77 (0.56)	1.83 (0.78)
FEV_1 % predicted, mean (std)	65.6 (16.6)	68.3 (21.4)
PDPE: physician defined pulmonary exacerbation Std: standard deviation IQR: interquartile range		

hmrM primer design

Designed using NCBI Primer BLAST and validated by performing qPCR on *H. influenzae* clinical isolate and non-*Haemophilus* controls.

Forward primer: GTGGAGAACCTGCACCCAAT

Reverse primer: AATTTGTTGCGAAGTGGCGT

Product length: 182 bp

Thermocycling conditions: 95°C for 30 s, 60°C for 60 s

Appendix 4: Supplementary Material from Chapter 5

The contents of this supplement have been prepared for publication as part of:

> **Taylor, S.L.,** Leong, L.E.X., Mobegi F., Choo, J.M., Wesselingh, S., Yang, I.A., Upham, J.W., Reynolds, P.N., Hodge, S., James, A.L., Jenkins, C., Peters, M.J., Baraket, M., Marks, G.B., Gibson, P.G., Rogers, G.B., and Simpson, J.L. Long-term azithromycin associated with altered airway microbiome and increased transmissible resistance gene carriage in severe asthma. *In preparation*

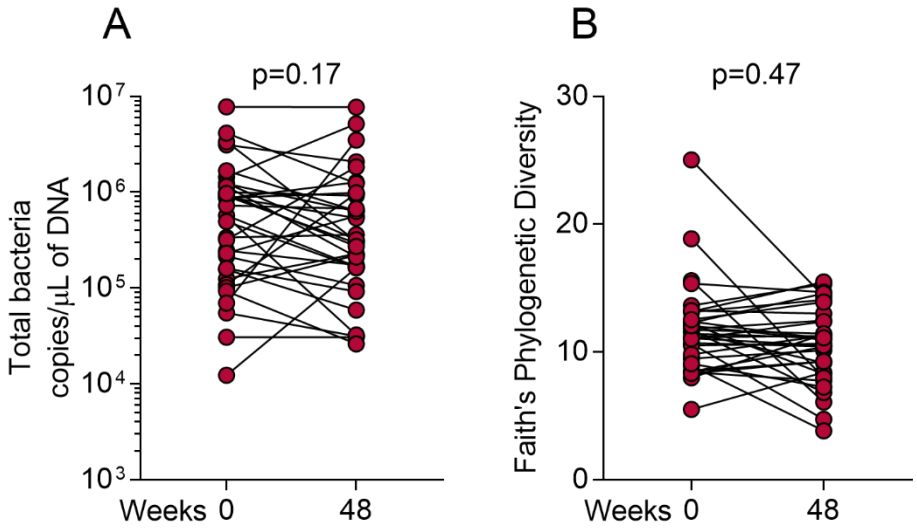

Figure E4.1: Bacterial load and microbiota composition in placebo. (A) Total bacterial load before and after trial in placebo group. (B) Faith's phylogenetic diversity before and after trial in placebo group.

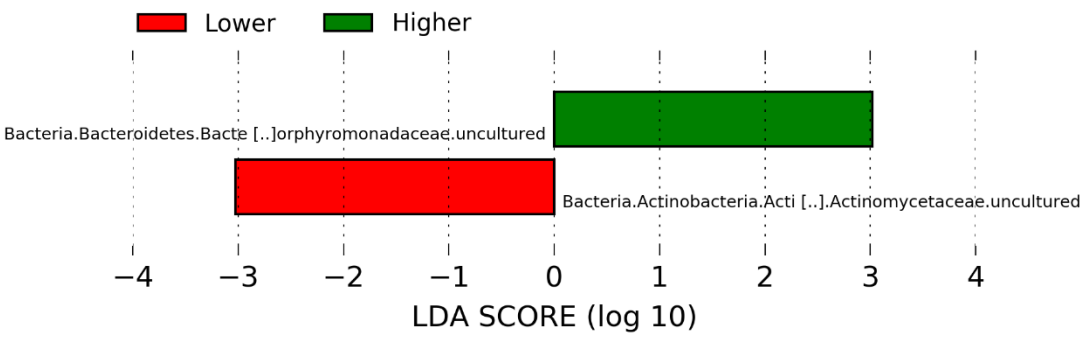

Figure E4.2: Change to individual taxa in placebo. Linear discriminant analysis effect size (LEfSe) of taxa that significantly changed in relative abundance before and after trial in placebo group. Red indicates taxa that were lower and green indicates taxa that were higher.

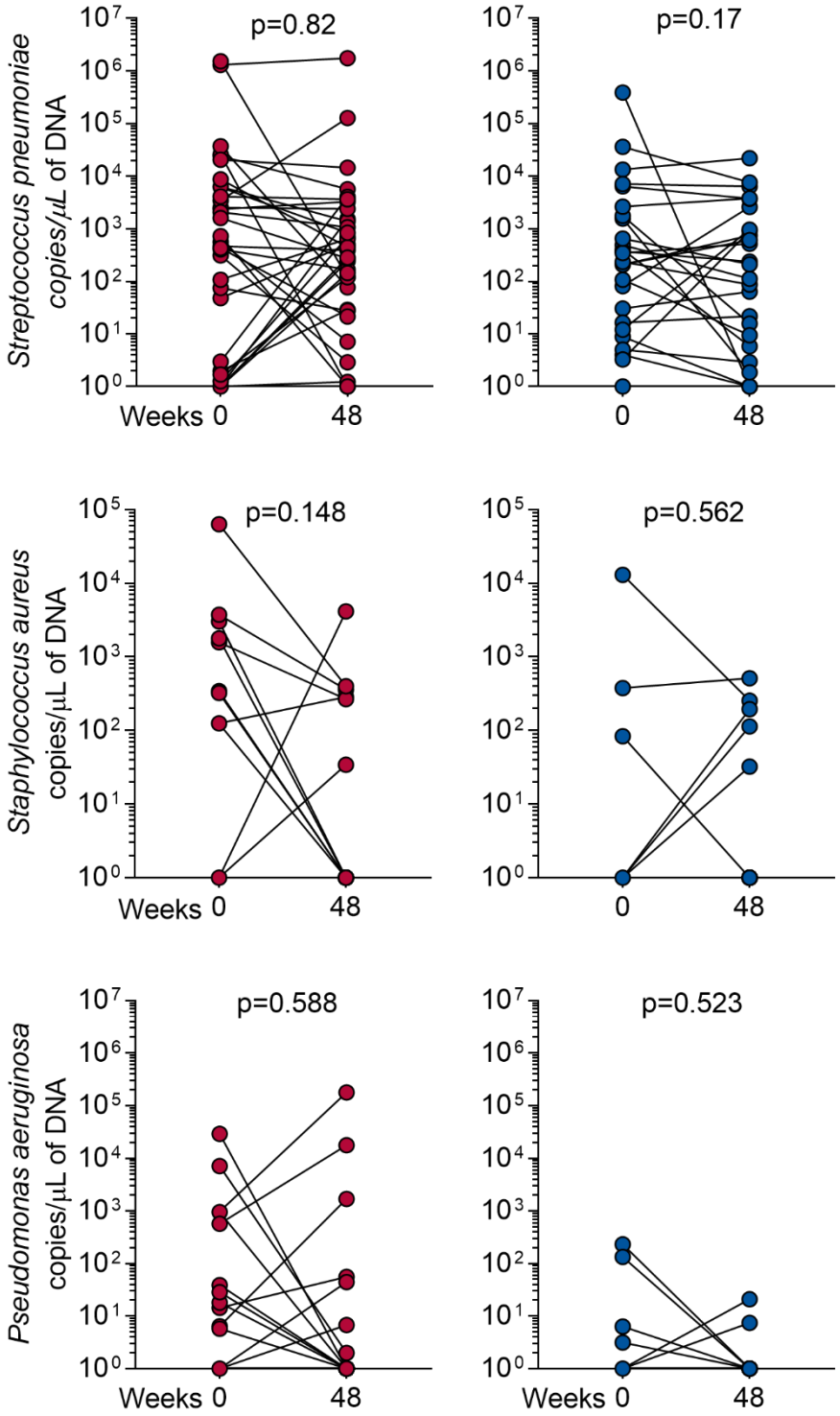

Figure E4.3: Effect of azithromycin on other pathogenic species. Copy number of *Streptococcus pneumoniae, Staphylococcus aureus* and *Pseudomonas aeruginosa* before and after either placebo (left) or azithromycin (right).

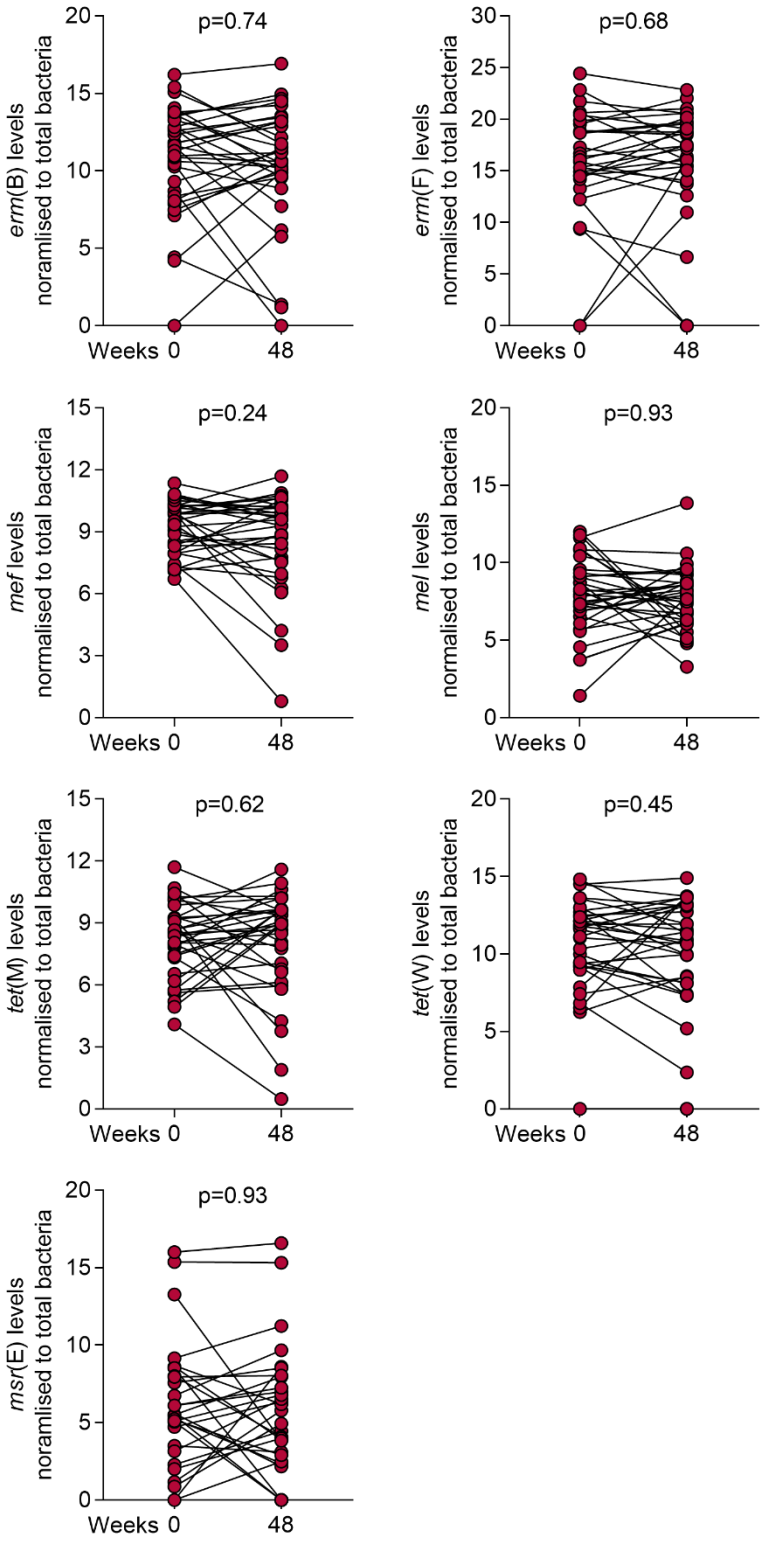

Figure E4.4: Levels of screened antibiotic resistance genes in placebo. Levels of the seven antibiotic resistance genes before and after trial in placebo group. Genes were identified by pooled-template shotgun metagenomic sequencing quantified by qPCR and normalized to total bacteria.

Table E4.1: Resistance genes and detected macrolide mutations from macrolide resistant isolates

AZM resistant species*	AZM zone (mm)	Week[a]	*erm*(B)	*mef*(A)	*mel*	*tet*(M)	*tet*(W)	*erm*(A)	*msr*(C)	L4 mutation[b]
Haemophilus parainfluenzae	13	0								
Haemophilus parainfluenzae	14	0								
Haemophilus parainfluenzae	8	48								G65A
Haemophilus parainfluenzae	13	48								G65A
Staphylococcus aureus	11	48						✓		
Staphylococcus aureus	6	48							✓	
Streptococcus australia	8	0		✓	✓					
Streptococcus infantis	11	0		✓	✓					
Streptococcus infantis	11	0		✓	✓					
Streptococcus mitis	6	0	✓			✓				
Streptococcus mitis	9	0		✓	✓					
Streptococcus parasanguinis	11	0		✓	✓					
Streptococcus parasanguinis	9	0		✓	✓	✓				
Streptococcus parasanguinis	9	0		✓	✓					
Streptococcus parasanguinis	11	0		✓	✓					
Streptococcus parasanguinis	9	0		✓	✓					
Streptococcus salivarius	7	0		✓	✓					
Streptococcus salivarius	8	0		✓	✓					
Streptococcus salivarius	6	0	✓			✓				

Streptococcus salivarius	11	0		✓	✓	✓				
Streptococcus anginosus group	8	48	✓			✓				
Streptococcus australia	10	48		✓	✓					
Streptococcus gordonii	9	48		✓	✓	✓				
Streptococcus infantis	9	48		✓	✓					
Streptococcus infantis	6	48	✓	✓	✓					
Streptococcus infantis	12	48		✓	✓	✓				
Streptococcus infantis	10	48		✓	✓					
Streptococcus mitis	8	48		✓	✓					
Streptococcus oralis	6	48	✓	✓	✓	✓	✓			
Streptococcus oralis	6	48	✓	✓	✓	✓				
Streptococcus oralis	6	48	✓			✓				
Streptococcus parasanguinis	6	48	✓	✓	✓	✓				
Streptococcus parasanguinis	10	48		✓	✓					
Streptococcus parasanguinis	6	48	✓	✓	✓					
Streptococcus parasanguinis	8	48		✓	✓					
Streptococcus parasanguinis	7	48		✓	✓					
Streptococcus salivarius	7	48		✓	✓					
Streptococcus sanguinis	10	48		✓	✓					

*Defined by EUCAST Clinical Breakpoints v.8.1. Where breakpoints are not defined, the epidemiological cut-offs were used
[a] Sputum sample collection week during azithromycin therapy
[b] Position numbers for L4 based on *H. parainfluenzae* numbering system