Airway Infection and *Pseudomonas aeruginosa* in bronchiectasis (cystic fibrosis and non-cystic fibrosis bronchiectasis)

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Overview

Bronchiectasis is a term used to refer to dilatation of the bronchi that is often permanent and associated a chronic productive cough and recurrent respiratory infections. It is caused by a wide range of clinical disorders, both inherited and acquired [1]. Bronchiectasis is not itself a disease, but rather the result of various processes that often share aspects of management.

A traditional distinction has been made between Cystic Fibrosis (CF) and non-cystic fibrosis bronchiectasis. CF is a genetic disease found in northern Europe and North America with a carrier frequency of 1 in 25 [2]. CF represents a more well-defined population of patients whom respiratory disease is the main predictor of mortality (2). It has subsequently been subject to more research and commercial activity than what has been undertaken in non-CF bronchiectasis. Non-CF bronchiectasis in contrast, affects a diverse heterogeneous population with many aetiologies, including idiopathic or unknown causes. The real incidence and prevalence of non-CF bronchiectasis in the general population is difficult to estimate as it is often underdiagnosed and shares many symptoms and disease characteristics with other respiratory illnesses. However, it is estimated 212,000 individuals have the disease in the UK [3].

Pulmonary exacerbations are defined as a deterioration in local symptoms such as cough, sputum production or purulence and breathlessness, with or without systemic upset, necessitating antibiotics [4]. Pulmonary exacerbations in CF and non-CF bronchiectasis are primarily driven by pulmonary infection and neutrophilic airway inflammation, which leads to progressive lung damage. However, evidence suggests other key factors can influence pulmonary exacerbations, disease progression and outcomes in both CF and non-CF bronchiectasis such as social deprivation, gender and environmental factors such as pollution [5]. Nevertheless, the treatment of pulmonary exacerbations and the management of respiratory infections in both CF and bronchiectasis present some of the greatest challenges in the modern age. Severe exacerbations requiring hospital admissions and intravenous antibiotics have

a greater impact in terms of healthcare costs as well as long term prognosis as these are associated with increased mortality, significant lung decline and reduced quality of life [6].

The true economic burden of bronchiectasis is not known, with only 5 published studies providing concrete figures. A study in 2013 of 456 bronchiectatic patients in Spain showed the mean annual cost per patient was €4671.9. Chronic bronchial infection with *P. aeruginosa* was the subgroup with the greatest impact on overall cost at 69.1% with an overall expenditure of €1,471,248 [7].

Many of the statistics we report both for CF and non-CF bronchiectasis are based on small studies and are unlikely to be representative or generalisable to the populations as a whole. Nevertheless, it is clear that both non-CF bronchiectasis and CF are placing an increasing burden on the healthcare systems internationally. Medical advances mean patients are living longer with chronic illnesses and the impact of recently approved therapies treating both the defect; as in the case with cystic fibrosis transmembrane conductance regulator (CFTR) modulator therapy in CF, or the underlying cause such as immunomodulatory therapy in asthma, is improving survival [8, 9].

P. aeruginosa is both an important and prevalent pathogen in both CF and bronchiectasis and is intrinsically resistant to many antibiotics. Persistent *P. aeruginosa* infection has been linked to poorer outcomes in non-CF bronchiectasis such as lung function decline and mortality similar to that reported in CF [10].

Nevertheless, there are a number of unknowns that span across the basic biology and pathogenesis of *P. aeruginosa* such as the interactions with other micro-organisms and the host defence at a cellular level, which cannot be answered with conventional microbiological techniques.

There is a need to develop greater understanding of the clinical impact of *P. aeruginosa* and other micro-organisms present in CF and non-CF bronchiectasis to define and develop optimal management strategies that is personalised to both the

patient and to their current microbiological status. A lack of high quality clinical data may be aided with better phenotyping of patient cohorts utilising severity scores, adverse outcomes such as lung function decline and mortality coupled with longitudinal studies exploring the whole pulmonary microbial community; termed microbiome, and intercellular signalling molecules [11] as biomarkers.

This thesis is divided into two parts. In the first part, clinical data from a cystic fibrosis observational cohort was obtained over an 8 year follow up period. Long term clinical outcomes such lung function decline, frequency of pulmonary exacerbations, mortality or lung transplantation were explored with *P. aeruginosa* quorum sensing (QS) molecules and anaerobic bacteria present in the sputum microbiota. In addition, the potential of QS molecules as biomarkers of *P. aeruginosa* burden were investigated by comparing QS molecules in sputum, blood, urine and saliva with pulmonary *P. aeruginosa* bacterial load measured by polymerase chain reaction (PCR).

In the second part, 50 participants with non-CF bronchiectasis who were experiencing a pulmonary exacerbation and requiring administration of intravenous antibiotics were recruited into a prospective observational study. The primary objective of this study was to explore the feasibility of collecting and analysing samples for *P. aeruginosa* quorum sensing (QS) molecules in this cohort. In addition, clinical data such as lung function, quality of life questionnaires, time to next exacerbation and mortality were obtained. However, due to the COVID-19 pandemic, data on quorum sensing molecules in non-CF bronchiectasis were unable to be analysed. Instead, sputum, blood and urine samples were used to explore the role of novel urinary inflammatory molecules and pro-inflammatory cytokines with pulmonary exacerbation status in individuals with non-CF bronchiectasis.

It was my aim in this thesis to explore both the clinical and microbiological factors that may influence our understanding of disease progression in CF and non-CF bronchiectasis. In summary, this thesis explores several novel non-microbiological strategies such as biomarkers as a non-invasive approach to better define treatment

responses in pulmonary exacerbations, predict adverse clinical outcomes and quantify the *P. aeruginosa* bacterial burden.

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Thank you to the Nottingham Biomedical Research Centre for funding this project and giving me such a fantastic opportunity. Rebecca Braybrooke and Jo Porte especially, for helping me with various administration tasks, invoices and ordering supplies (and our lunches). A massive thank you to Glenn Hearson for constructing the CRFs, databases, uploading data and being generally supportive. I would also like to thank Iain for all of the statistical support and for challenging me outside of my comfort zone. I would also like to thank Nottingham University Hospitals Charity who funded our microbiome/PCR and cytokine project through innovation grants.

I cannot explain how grateful I am to Laura Matthews and Helen Lee who taught and helped me process sputum, blood and urine samples and perform colony counts (even on Christmas eve!). They worked tirelessly to store and transfer samples, perform differential cell counts and print labels. I would not have been able to do this project or reach my recruitment limit without them. A special thanks to Carly Clayton and Laura Matthews who did the laboratory processing for the cytokine project.

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Declaration

For the cystic fibrosis observational cohort studies, the data collection, interpretation and analyses were done by myself.

In the bronchiectasis project, I recruited and obtained consent from all the participants and performed all of the visits (but 2) myself. I filled out all of the clinical information, the case report forms and reviewed all the patients' notes and historical records. I also submitted the IRAS document and obtained ethical approval for the bronchiectasis study. I successfully applied for 2 innovation grants from the Nottingham University Hospitals charity to fund the microbiome/PCR and cytokine projects.

In addition, I trained in the laboratory and, with the help of another scientist, processed over 95% of the biological samples obtained from the bronchiectasis study. However, the scientists carried out the differential cell counts and the cytokine analysis (Chapter 9).

For the Mologic ltd study (Chapter 8 and 9), I received 280 Headstart lateral flow assays and did all of the laboratory (with the help of a colleague) and data analysis myself.

Finally, I carried out all of the statistical analyses and drafting of manuscripts myself and I am either first or joint first author on all the corresponding papers related to this thesis.

COVID-19 IMPACT STATEMENT

Due to the COVID-19 pandemic, the non-CF bronchiectasis feasibility study for quorum sensing molecules was delayed by the time of writing this thesis. As such, further analysis was performed to explore the role of novel urinary inflammatory molecules and pro-inflammatory cytokines in this cohort.

Abbreviations

95%CI	95% confidence interval
AQ	2-alkyl-4(1*H*)-quinolone
BAL	Broncho-alveolar lavage
BMI	Body mass index
C7-PQS	2-heptyl-3-hydroxy-4(1*H*)-quinolone
C9-PQS	2-nonyl-3-hydroxy-4(1*H*)-quinolone
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator protein
CFU	Colony forming unit
CRP	C-reactive protein
FEV_1	Forced expiratory volume in one second
g	Gram
HHQ	2-heptyl-4-hydroxyquinoline
HQNO	2-heptyl-4-hydroxyquinoline-*N*-oxide
HR	Hazard ratio
IL	Interleukin
IQR	Inter-quartile range
IV	Intravenous
L	Litre
LCMS/MS	Liquid chromatography-mass spectroscopy
ml	Millilitre
NHQ	2-nonyl-4-hydroxyquinoline
NQNO	2-nonyl-4-hydroxyquinoline-*N*-oxide
non-CF bronchiectasis	Non-cystic fibrosis bronchiectasis
P. aeruginosa	Pseudomonas aeruginosa
PCR	Polymerase chain reaction
PIA	Pseudomonas isolation agar

QS	Quorum sensing
qPCR	Quantitative polymerase chain reaction
r	Spearman’s correlation coefficient
SD	Standard deviation
WBC	White blood cell
µg	Micrograms

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Chapter 1. General introduction

1.1 Non- CF bronchiectasis

1.1.1 Introduction

Bronchiectasis is a morphological term given to a lung condition when there is chronic dilatation of one or more bronchi. It is a progressive respiratory disease that incorporates both a permanent dilatation of the bronchi and presents with a high burden of symptoms. Until recent years it has been a relatively neglected disease but it is known to cause substantial morbidity to patients and is associated with increased mortality and reduced quality of life [12]. Non-CF bronchiectasis is now recognised at earlier stages due to an increase in clinician awareness and thoracic imaging, but despite this it still has a mortality of up to 20% in 5 years [13].

There is limited data available regarding epidemiology of this condition as it often shares symptoms with other respiratory diseases and some patients have a primary diagnosis of COPD or asthma meaning it remains underdiagnosed [14, 15]. It is characterised by structural damage to the lungs and is caused by a vast diversity of inherited or acquired conditions. A study conducted by Quint *et al*, investigated the changes in incidence, prevalence and mortality in bronchiectasis in the United Kingdom from 2004 to 2013 and found the prevalence in men was 486/100,000 and 566/100,000 in women [16]. Non-CF bronchiectasis is not only increasing in prevalence but it is more common in older age [17].

Persistent bacterial infection of the airway mucosa is a key driving event for the pathological processes associated with this condition and treatment decisions in bronchiectasis are hampered by the lack of evidence from randomised trials. A greater understanding both into drivers of pathogenicity and the appropriate management strategies for this heterogeneous condition are urgently needed.

1.1.2 Pathophysiology

It is important to note that there is no simple cause or mechanism to explain the pathophysiology in non-CF bronchiectasis as this chronic disease represents an endpoint of various causes with a complex interplay of inflammation, immune response and micro-organisms.

The "vicious cycle" hypothesis model was first described by Cole in 1986 [18]. This suggested that after an initial infectious event, compromised mucociliary clearance paves the way for micro-organisms to reproduce and persist in the airway. This then attracts more inflammatory cells which in turn release toxic factors that further damage the airways. This chronic inflammation subsequently reduces microbial clearance- hence the vicious cycle (Figure 1).

Neutrophils are crucial to the host response against micro-organisms where they degranulate their cytotoxic substances and immune molecules. In the airway, the presence of a prominent number of neutrophils along with chemotactic molecules such as CXCL-8 and leukotrienes LTB_4 are confirmed in sputum, bronchoalveolar lavage and in bronchial biopsies [19].

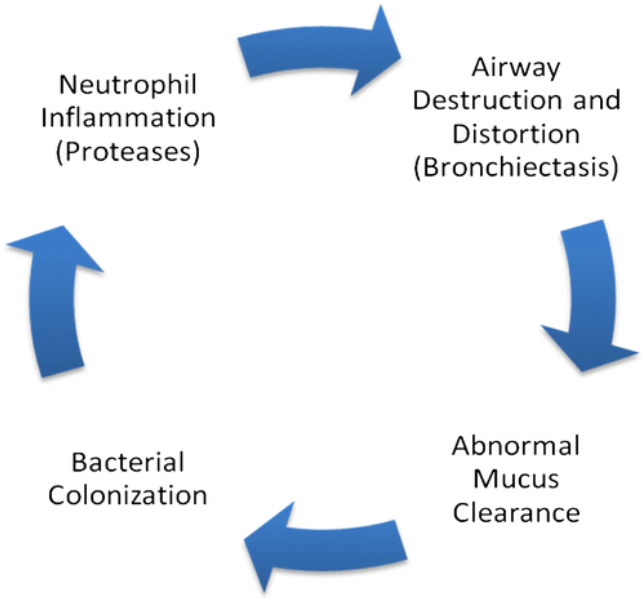

Figure 1-1. Vicious cycle hypothesis. A Host-mediated inflammatory response to foreign material and bacteria in the airway then causes tissue damage

resulting in airway dilatation, which contributes to abnormal mucus clearance and further bacterial colonization, image provided by Mcshane *et al* [20]. Reprinted with permission of the American Thoracic Society. Copyright © 2021 American Thoracic Society. All rights reserved.

A cross-sectional study by Dente *et al* [21], confirmed a higher number of neutrophils in the sputum in patients with chronic *P. aeruginosa* and correlated inflammation with severity scores such as the Bronchiectasis Severity Index [6].

Neutrophils isolated in the sputum of CF and non-CF bronchiectasis patients has been shown to exhibit ineffective phagocytosis. This may be explained by a higher concentration of Human Neutrophil Peptides (HNP) in the lung [22]. HNP are proteins stored in neutrophilic granules with antimicrobial activity, however, high levels of HNP could exert an inhibitory phagocytic activity (17). This may explain that despite the high levels of neutrophils in the lung, diminished phagocytosis may favour a scenario with inefficient bacterial killing and increased damage by release of proteases.

Proteases such as neutrophil elastase, play a key role in pathogenesis. Elastase can digest phagocytized bacteria but also are capable of destroying elastin, collaged, fibronectin, alpha1-antitrypsin and inhibitors of metallo-proteinases [23]. Additional cytotoxic properties also contribute to airway damage and reduces many innate defences and phagocytosis, facilitating *P. aeruginosa* infection [24].

1.1.3 Causes of non- CF bronchiectasis

Bronchiectasis is a clinical and radiological diagnosis due to a variety of innate and acquired diseases resulting in heterogeneous clinical pictures. As there are a large number of causes/aetiologies associated with non-CF bronchiectasis, a costly clinical and laboratory work up is required [25]. Even after extensive testing, the majority of patients are classified as idiopathic bronchiectasis [20, 26] (Figure 2).

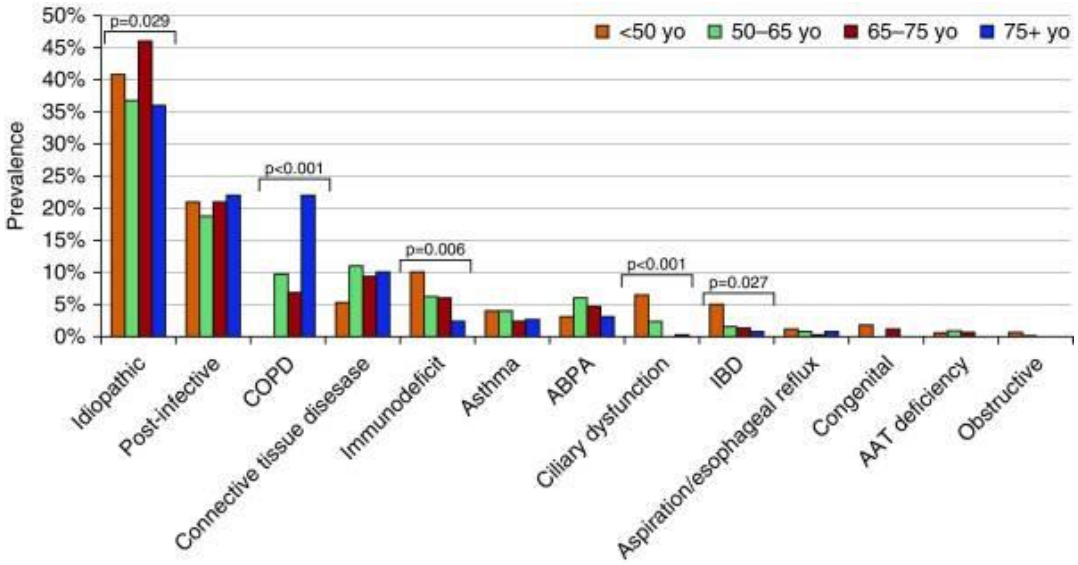

Figure 1-2. Aetiology of non-cystic fibrosis bronchiectasis according to age. AAT= alpha-1 antitrypsin, APBA= allergic bronchopulmonary aspergillosis, COPD= Chronic obstructive pulmonary disease IBD= inflammatory bowel disease, image provided by Lonni *et al* [27]. Reprinted with permission of the American Thoracic Society. Copyright © 2021 American Thoracic Society. All rights reserved.

Non-CF bronchiectasis is known to be a final common pathway in a number of various disorders and therefore it is essential to seek underlying causes. Standardised testing for causes leads to a change in treatment in up to 56% of cases [28]. Management that can be altered include replacement immunoglobulin therapy for combined variable immunodeficiency, anti-microbial treatment for non-tuberculous mycobacteria and the use of anti-fungals and corticosteroid medication for allergic bronchopulmonary aspergillosis [29].

1.1.3.1 Post infective

Post- infective bronchiectasis is a diagnosis made from a history of certain childhood infections such as tuberculosis, whooping cough, severe bacterial pneumonia and measles [27]. There has been a marked decrease in the prevalence of post-infective causes of bronchiectasis owing to the national vaccination programme (whooping cough and measles) and advances in medical treatment for infections [28].

1.1.3.2 Mucociliary disorders

Disorders that may lead to bronchiectasis include the failure of the mucociliary escalator which leads to bacterial airway infection and development of bronchiectasis. The most common disorder is Primary Ciliary Dyskinesia [30], which is a rare genetic disease. It is recommended that ciliary function tests should be made for all patients with chronic upper respiratory tract problems, male infertility and otitis media [4].

1.1.3.3 Immune deficiency

Any immune deficiency can be associated with bronchiectasis [19]. The British thoracic guidelines recommend that all patients should be screen for serum immunoglobulin deficiencies as well as functional responses to vaccination [4]. Immunodeficiency could be acquired such as HIV infection, malignancy, lung and bone marrow transplantation or primary such as hypogammaglobulinaema and other inherited immunoglobulin deficiencies (14).

1.1.3.4 Allergic Bronchopulmonary Aspergillosis (APBA)

APBA is caused by an excessive immune response to *Aspergillus fumigatus* which ultimately leads to airway inflammation and damage [31]. The generally recommended tests for ABPA are total serum IgE, and both specific IgG and IgE to Aspergillus [32]. Early treatment of APBA can prevent irreversible lung damage and testing is recommended for all patients with bronchiectasis (3). The treatment consists of a combination of corticosteroids and anti-fungal medication such as Itraconazole [29].

1.1.3.5 Non-tuberculous mycobacteria (NTM)

Non-tuberculous mycobacteria can be both a cause and also a direct complication of established bronchiectasis. The most common species are part of the *Mycobacterium avium* complex; *Mycobacterium avium* and *Mycobacterium intracellulare,* other less frequent isolates are *Mycobacterium* abscessus, *Mycobacterium kansasii, Mycobacterium chelonae* and *Mycobacterium fortuitum* [33].

Clinical features of NTM include haemoptysis, rapid weight loss and increase of symptoms. Treatment of NTM pulmonary disease is extremely difficult. Treatment regimens consist of multiple antibiotics with prolonged up to 18 months to 2 years [34].

1.1.3.6 Systemic inflammatory diseases

Inflammatory bowel diseases such as ulcerative colitis and Crohn's disease have been closely linked to non-CF bronchiectasis, although the exact pathological pathways are not known [35]. Interestingly, there is a clear relationship between curative colectomy and the diagnosis of non-CF bronchiectasis [35]. Connective tissue diseases such as Rheumatoid arthritis and Ankylosing spondylitis are important causes of non-CF bronchiectasis [36]. In fact, there is an association with worse outcomes in non-CF bronchiectasis and Rheumatoid arthritis [37]. Furthermore, it is not currently known if some systemic inflammatory diseases succeed or precede the development of non-CF bronchiectasis [38].

1.1.3.7 Other important causes

Cystic fibrosis (CF) is an autosomal recessive condition and is an important cause of bronchiectasis. It is often considered separately to other causes of non-CF bronchiectasis, due to the relatively young age of the individuals with CF and also the need for a multi-disciplinary approach to treatment that considers all the clinical manifestations of this disease.

Other causes include gastro-oesophageal reflux disease, which is the retrograde flow of acidic gastric contents into the oesophagus. Recurrent aspiration of gastric contents into the lungs causes lung damage and predisposes to non-CF bronchiectasis. Bronchial obstruction caused by foreign objects, tumour, lymph nodes or external compression are all known causes of non-CF bronchiectasis. Lastly, structural lung disease such as COPD and interstitial lung diseases can cause non-CF bronchiectasis.

1.1.4 Clinical aspects

1.1.4.1 Signs and symptoms

Non-CF bronchiectasis is a heterogeneous disease that presents with a wide range of clinical manifestations from asymptomatic to massive haemoptysis and respiratory failure.

The symptoms of non-CF bronchiectasis are a heavy burden and well recognised; chronic productive cough, recurrent chest infections, breathlessness, wheeze, haemoptysis, chest pains and extreme fatigue [39]. The most common symptom is cough, occurring in 82-98% of patients [40-42]. Dyspnoea is present in around 62-74% of patients, [43, 44] and is most prominent in patients with severe disease. Haemoptysis occurs in 26-51% of the cases and often presents in a mild fashion, but can result in shock, massive haemorrhage or respiratory failure if severe [40, 42]. Constitutional symptoms such as fatigue, malaise, recurrent fevers and decreased exercise tolerance are commonly encountered in non-CF bronchiectasis patients [40, 42].

Patients with mild disease may be asymptomatic, reporting symptoms only during exacerbations. Sputum colour may change over time and reflects the presence of bacterial infection, often being more purulent or darker during exacerbations [45, 46].

Rhinosinusitis symptoms such as rhinitis, sinus congestion and discharge are reported in up to 77% of patients [47]. The reason for these symptoms is multifactorial and may be related to common pathophysiologic mechanisms such as immunosuppression, ciliary dysfunction and mucus viscosity [47].

Physical examination often shows inspiratory crackles, diffuse rhonchi and prolonged expiratory due to airway obstruction and sputum blocking. Digital clubbing may be present in adults in 2-5% but is often higher in the paediatric population [48].

1.1.4.2 Radiology

Radiologically, non-CF bronchiectasis is defined by a characteristic bronchial dilatation on high resolution CT scan imaging, although in severe cases it may be detected by chest x-ray [4]. In healthy lungs, the diameter of a bronchus should be approximately equal to its adjacent pulmonary artery. Therefore, a ratio of bronchus diameter: vascular diameter >1 denotes abnormal dilatation [49]. Other features that are also associated with non-CF bronchiectasis are mucus plugging, bronchial wall thickening, segmental or lobar collapse and bronchiolitis [50].

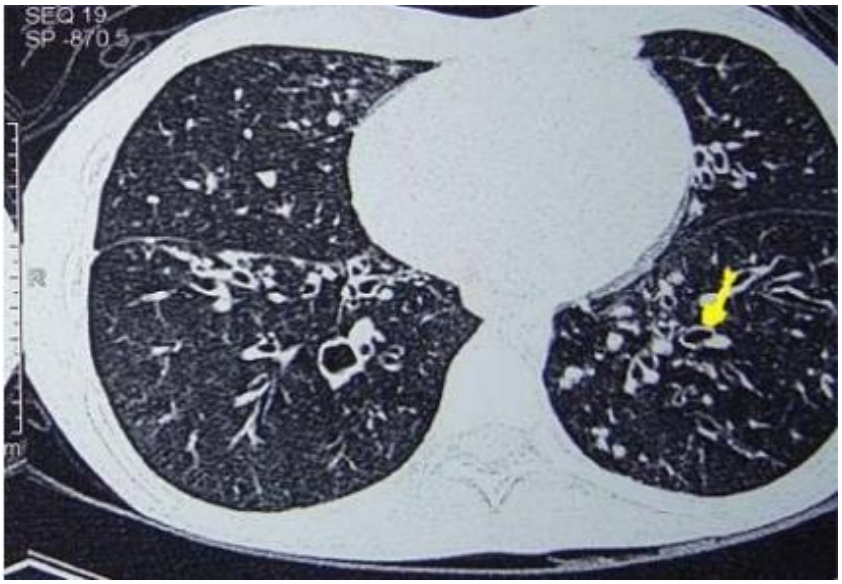

Figure 1-3. HRCT in a patient with cystic bronchiectasis showing dilatation of the bronchioles with thickening of their walls. Signet ring appearance depicted by yellow arrow, image by Ibraham *et al* [51]

1.1.4.3 Lung Function

Pulmonary function may be normal in patients with localized or mild non-CF bronchiectasis but the majority will have abnormalities on pulmonary function testing. Patients with diffuse non-CF bronchiectasis will typically have an obstructive pattern characterised by a reduced forced expiratory volume in 1 second (FEV_1) and a

reduced FEV_1 / forced vital capacity (FVC) ratio [52]. Patients may also have co-existing pulmonary fibrosis in association with their lung disease and may have a mixed obstructive/restrictive pattern in pulmonary function tests.

During a pulmonary exacerbation, the lung function will decline in some patients [53]. It has been shown that the degree of lung function impairment has implications in bronchiectasis patients. A study followed 197 patients prospectively after baseline lung function measurements. It reported that lower FEV_1 values were associated with a higher number of infectious exacerbations [42]. This study also showed that patients with a mixed obstructive/restrictive pattern on lung function also had a higher number of infective exacerbations compared with a normal function or obstructive pattern [42].

Lower FEV_1 and FVC values are noted to be correlated with the cystic type of non-CF bronchiectasis and with the presence of *P. aeruginosa* [54].

1.1.4.4 Quality of Life

Quality of life is one of the most prominent aspects affected by the presence of non-CF bronchiectasis. The huge burden of symptoms coupled with frequent exacerbations that often require multiple courses of oral, inhaled or intravenous antibiotics and hospital admissions all contribute to a poor quality of life. Furthermore, some patients may respond to pharmacological or non- pharmacological therapy, but a large proportion will remain symptomatic and treatment regimens add a further burden and take a significant amount of the patient's time [55]. Increased cough and sputum production may also contribute to a negative perception of a patient in the community and can lead to isolation, embarrassment and affect self-worth [56].

The presence of *P. aeruginosa* in sputum has been associated with a worse quality of life compared to other bacterial pathogens [57].

To evaluate quality of life, several questionnaires have been studied with the aim of providing value for therapeutic efficacy in clinical trials and providing a clinical took to assess treatment effect. This is relevant because other markers of severity or disease

such as FEV_1 do not always correlate with health status [58]. The Leicester Cough Questionnaire (LCQ) and the St George's Respiratory Questionnaire have been studied and validated for bronchiectasis, however the former focuses on cough and not the whole scale of symptoms that patients have with non-CF bronchiectasis and the latter was originally developed for COPD [59, 60]. For these reasons, the disease specific Quality of Life Questionnaire- Bronchiectasis (QOL-B) was developed and has been validated [61]. The QOL-B is an outcome tool that approaches all aspects of the disease burden in non-CF bronchiectasis. It emphasises on the patient's activities of daily living and symptoms on eight scales; respiratory, physical, role, emotional, social functioning, vitality, health perceptions and treatment burden [61]. A minor limitation to the QOL-B emerged from final psychometric analyses which found some patients had baseline scores of 100 on the emotional functioning scale, indicating that it would not be well suited to monitor improvements from baseline in a comparable study population [62]. Nevertheless, content validity, reliability and responsivity have been established both with cognitive testing and interview studies [63] and it is now considered the standard for evaluation of the health status of patients with non-CF bronchiectasis in routine clinical practice.

1.1.5 Severity Scores

Due to the heterogeneous nature of non-CF bronchiectasis, stratifying patients into mild, moderate and severe allows the clinician to assess mortality and morbidity and to tailor the correct treatment options to benefit each individual patient. Until recently, a validated model for assessing mortality and morbidity has not existed. Radiological imaging to assess severity in the past has not encompassed all aspects of disease severity [20].

1.1.5.1 Bronchiectasis Severity Index

The Bronchiectasis Severity Index (BSI) was developed by a team from Edinburgh using data from a 4-year prospective cohort study which included 1310 patients from other centres [6]. The end-points used in the assessment of this score were

hospitalisation rate, exacerbation frequency, mortality, and quality of life measures [6]. The BSI assigns points based on 9 variables allowing a 0-26 point score to be calculated, which stratifies patients to mild, moderate or severe disease. The variables which make up the severity index were found to directly impact primary outcomes, these were: age, body mass index (BMI), FEV_1 % predicted, hospital admission within the last 2 years, pulmonary exacerbation frequency in the last 12 months, MRC breathlessness score*, P. aeruginosa* colonisation, colonisation with other organisms and radiological severity [63].

A score of 0-4 equals mild non-CF bronchiectasis, a 4-year mortality rate and hospitalisation rate are 0-5.3% and 0-9.2% respectively. A score of 5-8 equals moderate non-CF bronchiectasis with a 4-year mortality and hospitalisation rate of 4-11.3% and 9.9-19.4% respectively. Lastly, a score of 9 or above equals severe non-CF bronchiectasis with 4-year mortality or hospitalisation rate of 9.9- 29.9% and 41.2-80.4% respectively (3). This assessment of severity was found to give excellent predictions of hospital admissions, quality of life and exacerbations. A separate validated scoring system to predict 5-year mortality is the FACED score and was first devised by Dr Martinez-Garcia [64]. The variables include FEV_1 % predicted, age, colonisation by *P. aeruginosa*, radiological extension of non-CF bronchiectasis, and the MRC scale. The FACED score has been designed to predict mortality and therefore it is not validated to assess likelihood of hospitalisation or exacerbations. A small study compared both scoring systems and found that the predictive power of mortality was similar [65] but in a large European study comparing the scoring systems, it was found FACED overestimated the mortality rate in severe non-CF bronchiectasis patients [66]. In comparison, the BSI was noted to accurately predict outcomes across all areas including exacerbations, quality of life, lung function decline, respiratory symptoms and hospitalisation [66]. Therefore, the BSI was used as a tool for predicting disease severity in my non-CF bronchiectasis observational study.

1.1.5.2 Bronchiectasis Aetiology Comorbidity Index (BACI)

The BSI was supplemented in 2016 by another scoring system by McDonnell *et al,* which assessed the impact that comorbidities have on a 5 year hospitalisation and mortality risk in non-CF bronchiectasis patients [67]. This cohort study analysed data from 986 patients to construct the BACI. Each comorbidity has associated points, the largest points attributed is 12 points for metastatic malignancy. Other comorbidities are: COPD, inflammatory bowel disease, iron deficiency anaemia, asthma, peripheral vascular disease, haematological malignancy, cognitive impairment, chronic liver disease, diabetes mellitus, pulmonary hypertension and ischaemic heart disease [67].

A calculated score of 0 is low risk, 1-5 is intermediate risk and high risk is >6 where estimated mortality and hospitalisation risk at 5 years 34.9% and 36% respectively [67]. As with the BSI, the BACI is validated to assess future mortality and exacerbation and hospitalisation risk. The authors showed that adjusting for co-morbidities strengthened the BSI score and together the scoring systems were superior when used simultaneously. Some limitations of the BACI scoring system international study is that it is relatively complex, the patients were younger and less co-morbid in one of the validation cohorts and so there may have been some recruitment bias and finally it may not have taken into account the different diagnostic criteria used in diagnosing comorbidities [67]. Furthermore, further studies are needed to determine how this score may impact clinical practice. It was also recommended by McDonnell *et al*, that it would be beneficial to explore the relationship between high BACI scores and lung or systemic inflammation in light of the association between higher exacerbations and *Pseudomonas* colonisation in co-morbid patients [67].

1.1.6 Pulmonary exacerbations in non-CF bronchiectasis

1.1.6.1 Definition

Like asthma and COPD, patients will suffer from an acute worsening of symptoms with a deterioration of respiratory status outside the normal day-to-day variation. Patients with frequent exacerbations have increased morbidity and mortality [68]. Until

recently there has been a need for a clear definition of exacerbations in non-CF bronchiectasis, for instance the British Thoracic Society Guidelines has defined exacerbations requiring antibiotics based on a deterioration of local symptoms and/or systemic upset [68].

The heterogeneity in the definition of exacerbation can hinder comparisons of treatment effect as exacerbation frequency and time to next exacerbation are key end-points in clinical trials. However, a recent systematic review carried out involving non-CF bronchiectasis experts form Europe, North America, Australia and South Africa has enabled a consensus definition to be used in clinical trials [69]. An exacerbation is therefore defined as deterioration in three of the following key symptoms for at least 48 hours and a clinician has determined that a change in treatment is required:

- Cough
- Sputum volume and/or consistency
- Sputum purulence
- Breathlessness and/or exercise tolerance
- Fatigue and/or malaise
- Haemoptysis

1.1.6.2 Aetiology of pulmonary exacerbations

Pulmonary exacerbations cause a high impact on quality of life and long-term prognosis for non-CF bronchiectasis and it is assumed that exacerbations are mostly due to infections, although air pollution and low social-economic status is also a risk factor [70, 71].

A recent study by Rosales *et al*, showed *P. aeruginosa*, respiratory viruses, *Staphylococcus aureus*, *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catharrhalis* to be most frequent micro-organisms isolated from sputum cultures or swabs (PCR analysis of viruses) of exacerbated patients [72]. In addition, patients suffering from pneumonia, *S. pneumoniae* continues to be the most frequent

isolate, irrespective of airway chronic inflammation and interestingly atypical pneumonic infections are uncommon [72]. A combination of bacteria and virus was found in 22% of cases of pneumonic infection and 11% in non-pneumonic exacerbations [72].

The role of viral infections in exacerbation has been confirmed in previous studies which showed viruses are present in up to 50% in both adult and children populations [73, 74]. Indeed, Gao *et al*, showed that virus positive exacerbations were associated with higher inflammatory markers compared to virus negative exacerbations, and those patients with a virus present, were more likely to go onto intravenous antibiotics [73]. The most common viruses detected are coronavirus, rhinovirus, influenza A and B, metapneumovirus, respiratory syncytial virus and parainfluenza 3[74]. A human model of rhinovirus infection has showed an impairment of IFN production and neutrophilic inflammation which can trigger a COPD exacerbation through inflammatory mechanisms [75]. Alternatively, viruses are able to induce secondary bacterial infection in COPD [76]. However, these mechanisms of pathophysiological patterns have not clearly been demonstrated in bronchiectasis. Changes in the host-pathogen interaction could be hypothesized during exacerbations due to current evidence on microbiome, classical microbiology and inflammatory patterns [73, 74, 77]. The lung microbiome has been shown not to change significantly between exacerbations and clinical stability [78].

1.1.6.3 Treatment of exacerbations

There is clear scientific and clinical evidence that antibiotics decrease both the airway bacterial load and systemic and local inflammation in blood and sputum [79]. This is reflected in available guidelines which suggest treating exacerbations with 14 days of antibiotic therapy [4, 80, 81]. Systemic antibiotic therapy is recommended by the oral or intravenous route with the choice of therapy dependant on recent or past microbiological culture results, whether the infection is pneumonic or non-pneumonic and taking into consideration the severity of the exacerbation. However, as a result of widespread use of antibiotics, the treatment of pulmonary exacerbations is often

hindered by antibiotic allergies, intolerances and the emergence of resistant bacterial pathogens in non-CF bronchiectasis [82]. Inhaled antibiotics are not currently recommended to treat exacerbations due to the poor tolerability with regards to coughing, wheezing and bronchospasm.

H. influenzae exacerbations are normally treated with amoxicillin-clavulanic acid, doxycycline or a fluoroquinolone such as ciprofloxacin or levofloxacin. However, there are limited oral options to treat *P. aeruginosa* infections; such as ciprofloxacin. Therefore, intravenous antibiotics (IV) such as ceftazidime, meropenem, aztreonam or piperacillin-tazobactam are frequently required. There is a notable lack of scientific evidence, but in patients with mucoid multi-drug resistant *P. aeruginosa* strains, combination therapy with aminoglycosides or colistin is often used in clinical practice.

1.1.6.4 Prevention of Exacerbations

The use of chronic oral macrolides and inhaled antibiotics has widely been supported by experts worldwide. There have been three randomized clinical trials (EMBRACE, BAT and BLESS) that has shown a clear reduction of exacerbations with use of azithromycin three times a week or continued use of erythromycin [83-85]. Inhaled antibiotics achieve a high local concentration in the airways with limited occurrence of antibiotic resistance and are shown to prolong median time to exacerbation [86-88]. Inhaled antibiotics available include ciprofloxacin, levofloxacin, aztreonam, colistin, gentamicin and tobramycin. Furthermore, recent European guidelines recommend the use of macrolides and inhaled antibiotics to prevent exacerbations of patients known to be colonized with *P. aeruginosa* [81].

1.1.7 Bacteriology

1.1.7.1 Overview

Diverse polymicrobial communities are present in the airways of patients with bronchiectasis [78]. Studies have shown that the most common organism isolated in bronchiectasis patients is *H. influenzae* [52, 89, 90]. However, *P. aeruginosa* is the

organism that presents the greatest challenge in this patient cohort and is associated with a worse prognosis [6, 64]. Epidemiology studies have shown that approximately 20-35% of patients worldwide with non-CF bronchiectasis are chronically infected with *P. aeruginosa* [52, 89, 91].

Other gram negative organisms that can cause pulmonary infection in bronchiectasis patients include: *Strenotrophomonas maltophilia*, *Klebsiella pneumoniae*, *Moraxella catarrhalis*, *Achromobacter*, *Serratia marcescens* and *Eshcerichia coli.* If these organisms are isolated repeatedly then it is advised to target antibiotic therapy appropriately [92].

Gram positive organisms include *Staphylococcus aureus* and *Streptococcus pneumoniae.* The US bronchiectasis registry showed approximately 12% of patients had a sputum culture positive for *S. aureus* (both methicillin susceptible and methicillin resistant strains) and 3% *S. pneumoniae* [91].

Other organisms to note are non-tuberculous mycobacteria (NTM) and Nocardia [91], which are particularly challenging to treat and diagnose. It is recommended to treat only repeatedly positive cultures of these organisms as not all patients require antibiotic therapy for these conditions.

Fungal pathogens such as Aspergillus and Candida are commonly found in respiratory secretions but often these organisms can be isolated in the absence of disease, for example, Aspergillus may be an environmental contaminant and Candida is often associated with oral thrush. However, disease is associated with Aspergillus, such as allergy (ABPA or sensitisation) or infection (aspergillus bronchitis, aspergilloma, chronic invasive aspergillosis).Therefore, careful evaluation of the patient's symptoms, serial microbiological surveillance, immunology and radiology interpretation are often needed to ascertain if Aspergillus is having an adverse impact on the patient [93].

Micro-organisms other than *P*. *aeruginosa* have a significant impact on patients with bronchiectasis. Nevertheless, the full complexity of the polymicrobial lung

environment in non-CF bronchiectasis and the impact on disease is still not fully understood. Careful microbiological surveillance is needed in patients with non-CF bronchiectasis both in the stable and exacerbated states so the correct treatment can be tailored to the individual patient.

1.1.8 Pseudomonas aeruginosa

1.1.8.1 Introduction

P. aeruginosa is a gram-negative rod that grows readily in aerobic conditions but is a facultative anaerobic organism and can therefore achieve growth in the absence of oxygen. This is highly relevant within the mucus plugs seen in bronchiectasis where anaerobic conditions are expected to occur [94]. *P. aeruginosa* is an opportunistic pathogen that causes acute invasive infections in immunocompromised individuals and chronic persistent pulmonary infections in patients with CF and non-CF bronchiectasis [95]. However, it has been more widely studied in relation to chronic lung infections of patients with CF [96].

P. aeruginosa is the second most common pathogen in non-CF bronchiectasis and in some studies, it is present in up to 40% of adult patients [6, 97, 98].

The prevalence of *P .aeruginosa* in non-CF bronchiectasis is less common compared to the CF population. However, with over 100,000 of patients affected with non-CF bronchiectasis in the UK [16], this potentially means up to 40,000 patients may have persistent *P. aeruginosa* infection. This is a sharp contrast to CF, as 10,000 individuals are affected with CF in the UK and approximately 3,500 will be infected with *P. aeruginosa* by adulthood [99]. This means *P. aeruginosa* in non-CF bronchiectasis may be a huge and unrecognised health care burden, furthermore, there are relatively few longitudinal studies of non-CF bronchiectasis and *P. aeruginosa*.

To facilitate progression from an acute to chronic infection, *P. aeruginosa* produces distinct virulence factors [100]. *P. aeruginosa* isolated from patients with acute

respiratory infections generally are non-encapsulated and express a variety of invasive virulence factors such as: flagella, type III secretion system /9T3SS), type IV pili (TFP) and multiple secreted toxins and degradative enzymes [100].Other important virulence factors that contribute to pathogenicity are proteases and elastase (or LasB protease), which are known to cause extensive tissue damage during infection [101, 102] and iron-chelating siderophores, such as pyoverdine [103]. Rhamnolipid has biosurfactant properties and is a key component in the protection against host immunity [104] and pyocyanin, a blue redox- active metabolite which mediates tissue damage and necrosis [105].

The available data suggests that there are multiple phenotypes in *P. aeruginosa* in non-CF bronchiectasis with regards to mucoidity, hypermutability and antibiotic resistance [100, 106, 107]. However, fundamental differences between isolates from CF and bronchiectasis are not fully understood. Nevertheless, *P. aeruginosa* elicits an inflammatory response in the host; such as interleukin-8 which drives airway neutrophilia [108], and matrix metalloproteinases which may have a role in disease progression [46, 109].

Epidemic strains of *P. aeruginosa* in CF populations have led to strict infection control guidelines to prevent transmission. A few small studies have found no single dominant ‘epidemic strain’ in molecular typing of *P. aeruginosa* in non-CF bronchiectasis [110, 111]. It is clear that larger scale multicentre studies are needed to explore the molecular epidemiology of *P. aeruginosa* in non-CF bronchiectasis and how these may compare with CF epidemic strains. A recent study from the UK using whole genome sequencing of 189 isolates showed similar strains may be shared between non-CF bronchiectasis patients, raising the question of transmission in this population [112]. If confirmed, this may have important infection control repercussions for non-CF bronchiectasis patients both in hospital and the outpatient settings.

Outcomes in non-CF bronchiectasis patients infected with *P. aeruginosa* include greater levels of airway inflammation, increased hospitalisation, higher morbidity

and mortality [10, 113, 114]. A systemic review of observational studies showed that *P. aeruginosa* is associated with an almost seven-fold increase in risk of hospital admission, a three-fold increase in mortality risk and an average of one additional exacerbation per patient per year [10]. *P. aeruginosa* is intrinsically resistant to many antibiotics due to the presence of efflux pumps; such as MexAB- OprM and MexCD-OprJ [115], which function to remove antibiotics from the cell, enzymes that deactivate antibiotics, such as AmpC, [116] and transport proteins, such as porins (OprD), [117] preventing antibiotic entry. However, in contrast to CF, multidrug resistance and pan resistance appear less common, 45% of isolates are resistant to one antibiotic but multidrug resistance is rare [97]. A variety of antimicrobial sensitivity patterns can be seen in different isolates from the same individual and may represent phenotypical heterogeneity of the population [97, 118]. It is unclear if this situation mirrors CF in the respect that antibiotics may have *in vitro* resistance but may continue to benefit the patient clinically and hence questions the use of *in vitro* sensitivities in predicting clinical response to therapy.

1.1.8.2 Eradication therapy

Clinicians often attempt 'eradication' of *P. aeruginosa* in non-CF bronchiectasis early after isolation, however eradication is highly empirical and successful outcomes are problematic to measure [98, 119]. The success of eradication may be dependent on early detection of *P. aeruginosa* but clinical services and sampling regimes may not be sufficiently frequent enough to capture early acquisition events. Despite recommendation that sputum is sampled at every clinic visit, nearly a third of clinicians only sampled sputum during exacerbations [119]. Eradication according to the European guidelines of non-CF bronchiectasis is defined as achieving complete clearance of the pathogen from the airway [81]. The recent recommendation suggests that a new isolation of *P. aeruginosa* in a non-CF bronchiectasis patient, should be offered eradication treatment, although the quality of evidence behind this is low.

Commonly used regimens include oral ciprofloxacin plus 3 months of nebulised colistin and occasionally intravenous antibiotics [68]. The BTS guidelines note the lack of evidence and subsequent recommendations in this area are based on expert opinion. BTS suggest 2 weeks of oral ciprofloxacin and IV antibiotics or 3 months of nebulised antibiotics if this fails [68]. The SEPAR guidelines suggest 3 weeks of oral ciprofloxacin plus nebulised antibiotics for 3-12 months [80]. The recent European guidelines suggest nebulised antibiotics achieve greater clearance rates compared to intravenous treatment alone [120].

Eradication by many clinicians is judged by 2-3 sputum cultures negative for *P. aeruginosa* over a 12 month period [120]. However, it is not clear how clinicians define new acquisition of *P. aeruginosa* and when 'chronic infection' is established. No definition exists in current BTS guidelines, whilst SEPAR defines chronic infection as three of more positive cultures of *P. aeruginosa* within a 6-month period [80]. Therefore, the studies reporting eradication to date have all used various definitions [121, 122] and there needs to be an internationally accepted definition to help explore the success rate of eradication therapy.

There is no randomised controlled trial to address the question of eradication therapy in non-CF bronchiectasis. Two studies that investigated eradication treatment in adult patients with non-CF bronchiectasis showed some benefits such as lower frequency of exacerbations and improved quality of life, although the evidence of this is indirect [121, 122]. For instance, White *et al* performed a retrospective study and the results demonstrated eradication in 80% of patients with a maintained clearance rate of 50% at 12 months. However, there was a small number of patients studied and treatment regimens varied and it was prone to selection bias [122]. Furthermore, Orriols *et al* showed that 15 days of IV tobramycin plus ceftazidime followed by 3 months of nebulised tobramycin led to an eradication in 90.9% with a maintained eradication in 54.4% at 15 months [122]. However, this study excluded mucoid strains of *P. aeruginosa*, which is associated with a lower success rate of eradication treatment (100).

Most studies investigating *P. aeruginosa* eradication have a finite time-point with evidence suggesting that many people re-acquire *P. aeruginosa* in the future [123]. Perhaps the most appropriate aim for *P. aeruginosa* eradication should be to achieve 'maximal bacterial suppression' with prolongation of time without *P. aeruginosa* as the ultimate realistic goal. Prospective randomised control trials of how to manage new acquisition of *P. aeruginosa* is a key priority for both patients and clinicians [124].

1.1.8.3 Maintenance long-term therapy for P. aeruginosa

The management of chronically infected individuals with *P. aeruginosa* will often focus on maximising bacterial suppression in an attempt to reduce exacerbation frequency. Both BTS and SEPAR guidelines [68, 80] recommend long term therapy for those patients with recurrent exacerbations in the presence of a pathogen and clinical disease burden. BTS suggests an exacerbation frequency of three or more a year as a threshold for treatment [68]. Larger trials have undergone meta-analyses which have demonstrated the consistent effect of macrolides in reducing exacerbation and improvement in quality of life [125, 126]. There was a range of pathogens included in these trials, however subgroup analysis showed eradication of *H. influenzae* and a treatment response with *P. aeruginosa*. Furthermore, recent data supports the role of macrolides as inhibitors of LasR and *pqsA* quorum sensing regulatory genes from *P. aeruginosa* isolates in non-CF bronchiectasis [127].

There are no trials comparing macrolides to nebulised antimicrobials. One recent meta-analysis that looked at a variety of nebulised antimicrobial agents including: colistin, gentamicin, amikacin, tobramycin, ceftazidime and inhaled dry-powder ciprofloxacin, showed patients in the trials were four times more likely to eradicate their baseline pathogen, including *P. aeruginosa* compared to macrolides [128]. Nevertheless, to date, all antibiotics used as suppression therapy, either oral macrolides or targeted nebulised are 'off label' thereby reflecting no specific licencing studies have been conducted in non-CF bronchiectasis like CF.

Additionally, in selected patients who have severe airflow limitation and a high symptom burden, clinicians have adopted cyclical intravenous antibiotics in an attempt to suppress bacterial load [129]. Similarly, there are no high quality randomised control trials published in this area and it is unclear if dual agent therapy should be used to minimise bacterial resistance. However, many clinicians have seen an individual patient benefit using this technique despite the lack of published data. This is based on clinical outcomes such as a reduction in exacerbation frequency or overall clinical wellbeing. A recent consensus opinion identified the need for a simple biomarker that will help define a measurable treatment effect during the initiation phase of long-term suppression therapy [98].

1.1.8.4 Summary

Our understanding of the pathogenicity and treatment of *P. aeruginosa* in bronchiectasis is currently limited, for instance, the risk factors for acquiring this pathogen and the persistence of initial infection leading to chronic infection are not known. There is also no robust data on the use of dual antimicrobial therapy verses monotherapy and the duration of IV antibiotic therapy. Current guidelines recommend 14 days but this is based on expert opinion rather than robust evidence [119]. Furthermore, the optimal management strategies for established *P. aeruginosa* infections are also mainly based on expert opinion. The use of inhaled antibiotics, which take time to administer, side effects of frequent oral antibiotics and hospital admissions for IV antibiotics all contribute to an impaired quality of life. The management and diagnosis of *P. aeruginosa* infection in non-CF bronchiectasis is increasingly recognised as a key research priority.

1.1.9 Treatment Endpoints

The objectives of treatment of non-CF bronchiectasis are to improve respiratory symptoms, quality of life and reduce exacerbations. When an underlying cause is identified, it is recommended that it should be treated. However, the vast majority of patients have idiopathic non-CF bronchiectasis or aetiologies that cannot be

specifically targeted such as primary ciliary dyskinesia [27]. Efforts should be made to disrupt the circle of non-CF bronchiectasis consisting of the initial inflammatory insult resulting in airway dilatation, with subsequent impairment of mucus clearing, resulting in infection and increasing inflammation. Patients with active infective exacerbations should be treated with the appropriate anti-microbial therapy based on recent or past microbiological results.

There is evidence to suggest that patients with recurrent exacerbations may benefit from long-term antibiotic use such as macrolides, which have a potent anti-inflammatory and immunomodulatory effect [84].

Airway clearance techniques are encouraged to prevent mucus retention in the dilated airways, thereby improving sputum expectoration, quality of life and pulmonary function [130]. Pulmonary rehabilitation may also improve quality of life and exercise capacity in patients with advanced disease [131]. As a last resort, surgical intervention may be considered for life-threatening haemoptysis not responding to medical therapy [132]. Lastly, bilateral lung transplantation is an option for those with severe underlying pulmonary disease when all therapeutic strategies have been optimised.

1.2 Cystic Fibrosis

1.2.1 Introduction

Cystic fibrosis (CF) is a multisystem disorder caused by defective chloride regulation resulting from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) protein that regulates chloride transport in and out of epithelial cells. It is characterized by abnormal chloride transport across epithelial cells leading to increased viscosity of mucus. The pathophysiology of defective CFTR function is complex but ultimately an accumulation of muco-purulent secretions and defective mucociliary clearance lead to airflow obstruction and inflammation in CF airways

(Figure 4). This process contributes to chronic airway infection and inflammation which is the leading cause of morbidity and mortality in CF.

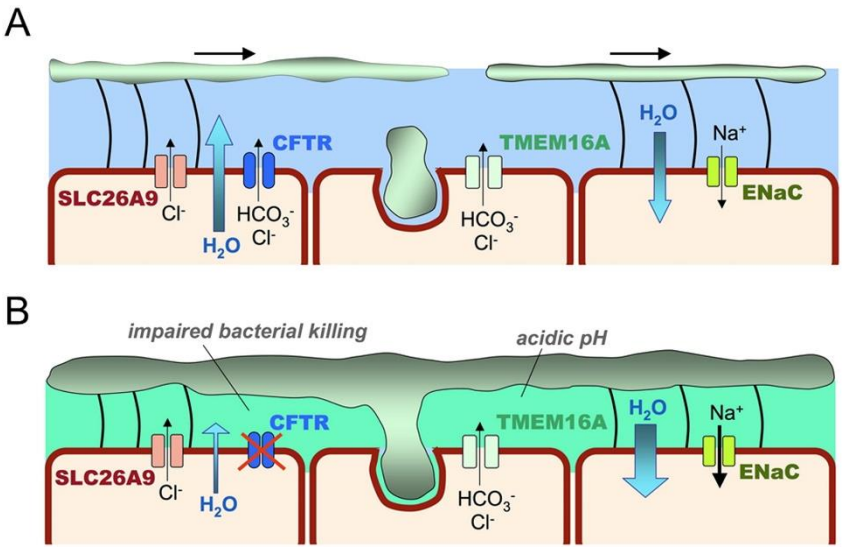

Figure 1-4. Epithelial ion transport defects and impaired host defence in CF airways. (A) In healthy airways, a coordinated secretion of salt and water driven by CFTR in conjunction with the alternative chloride channels; TMEM16A and SLC26A9, and absorption by the epithelial sodium channel ENaC, result in proper hydration of the airway surface layer (ASL) that is essential for effective mucociliary clearance. Further, CFTR-mediated bicarbonate secretion contributes to pH regulation of airway surfaces. (B) In CF airways, CFTR malfunction impairs secretion of chloride/fluid and bicarbonate leading to dehydrated and acidic ASL and hyperconcentrated mucus. ASL dehydration is further aggravated by increased ENaC-mediated sodium/fluid absorption. As a result, mucociliary clearance and bacterial killing are impaired making CF airways vulnerable for infection and inflammation. Image from Mall *et al* [133].

It is estimated that there are between 70,000 and 100,000 people with CF worldwide and around 10,600 people in the UK have cystic fibrosis [99, 134]. The gene encoding CFTR is located on the long arm of chromosome 7 and is inherited in an autosomal recessive manner [135]. There have been up to 2000 mutations in the CFTR gene identified [136]. The most common mutation is Phe508del, which ultimately results in defective processing of the CFTR [137]. The dehydrated thickened secretions in the airway; resulting from excessive salt and water reabsorption, allow a favourable environment for the development of bacterial niches in the airways [138]. Interestingly, beyond CF lung disease, CFTR- related cellular mechanisms that

regulate mucociliary clearance have been recognised in cigarette smoke-induced COPD [139].

It is known that infection, persistent inflammation and periodic episodes of acute pulmonary exacerbation all contribute to an irreversible decline in lung function [140]. The gradual ongoing destruction of the lung architecture secondary to inflammation in response to chronic infection is the major contributor to a shortened life span in the CF cohort compared to the general population. Predicted median life expectancy for people with CF is now reaching into the 5th decade [141]. Increasing understanding of how to prevent established chronic pulmonary infection has led to much of the improvement in life expectancy. Advances in antimicrobial therapy have contributed significantly to increased life expectancy. However, this comes at a cost of increasing multi-drug resistant organisms and new emerging pathogens.

1.2.2 Pathogenesis of pulmonary infection

Pulmonary infection begins early in life and over time becomes chronic in many patients. The infection results in an immune mediated inflammatory response which promotes the release of pro-inflammatory mediators [142]. The neutrophil inflammatory response is partially effective in the initial antibacterial defence by phagocytosing bacteria and releasing or activating enzymes such as peroxidases and proteases (including neutrophil elastase) [143]. However, with repeated episodes of infection, in the context of reduced mucociliary clearance, bacteria have the opportunity to proliferate and form biofilms. This subsequently means that inflammation in the airway becomes chronic. Overproduction of neutrophil elastase in the airways will exceed anti-protease binding capacity and be capable of elastin digestion, which is a presumed to underlie the development of bronchiectasis [144]. Furthermore, the proteases and oxygen radicals impair the innate immune function, resulting in reduced phagocytosis [145]. The stimulated immune system therefore in turn promotes the destructive inflammatory process in the lung, resulting in airway wall thickening with dilatation of the medium and small airways [136].

1.2.3 Bacteriology

Newer culture independent techniques in recent years such as microbiome analysis have shed further light into the complexity of the airway bacterial flora in this population of patients. Culture techniques are increasingly recognised as presenting merely the tip of the iceberg of bacterial pathogens in the airways. Nevertheless, historically, the bacteria cultured from airway secretions from people with CF come from several distinct genera (Figure 4). Early in life, *Staphylococcus aureus*, either methicillin resistant (MRSA) or sensitive (MSSA), is the commonest organism isolated [146]. *Haemophilus influenza*, *Stenotrophomonas maltophilia* and a range of other gram negative infection can also cause infection. Into the second decade, *P. aeruginosa* becomes established as a chronic infection, the incidence increases throughout childhood from approximately 20% at 1 year old to 80% of adults in their late twenties [147].

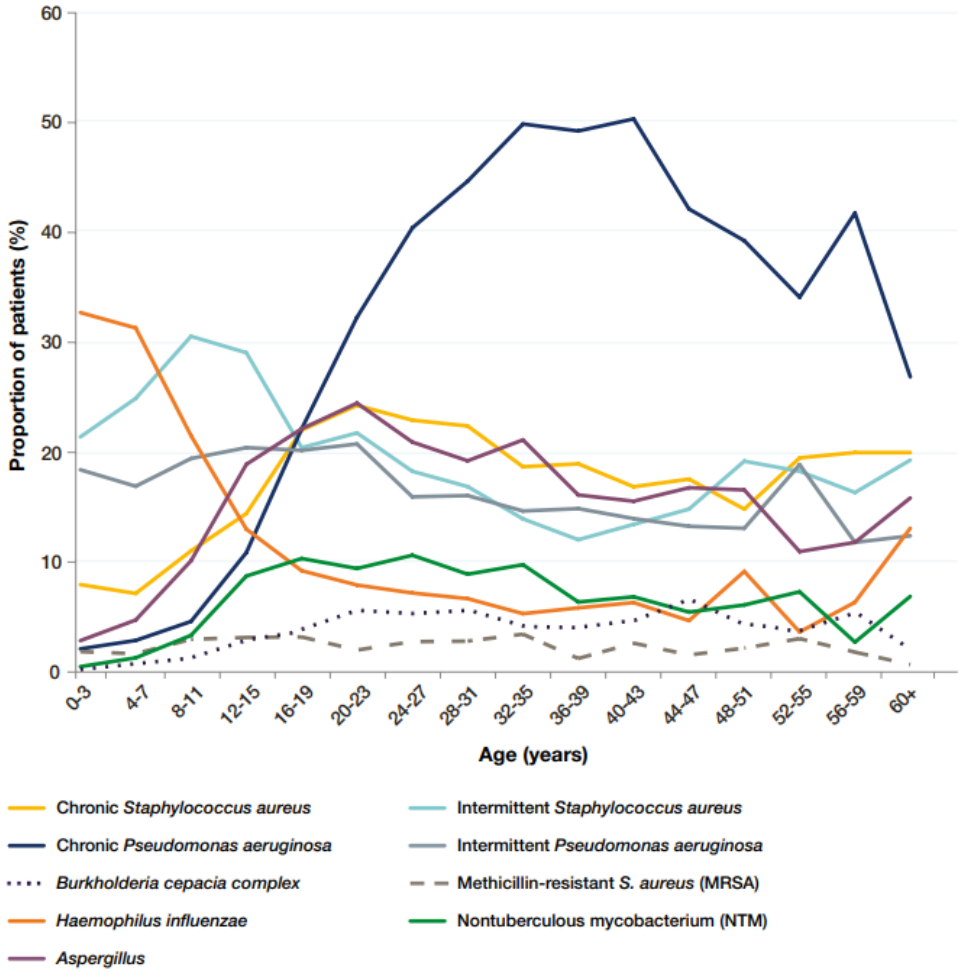

Figure 1-5. Lung infection prevalence by age in people with CF in the UK for 2019 Taken with permission from the Annual Data Report 2019 of the UK Cystic Fibrosis Registry [99].

Bacteria from the *Burkholderia cepacia complex*, most particularly *B. cenocepacia, B. multivorans* and *B. dolosa* have all been associated with outbreaks and in the case of *B. cenocepacia* or *B.dolosa*, are also associated with reduced survival [148]. In CF, these organisms are known to cause a fatal necrotising pneumonia, worsening respiratory failure and bacteraemia, known as Cepacia syndrome [149]. In addition, they are intrinsically resistant to many antimicrobial agents and have increased virulence, making them extremely problematic [150].

Other organisms such as *Achromobacter xyloxidans* and *Stenotrophomonas maltophilia* are opportunistic pathogens [151] and are often selected out due to their high resistance to most antimicrobial agents, presenting significant challenges in terms of treatment.

Non-tuberculous mycobacteria (NTM) has been increasingly recognised since the 1990s [152]. NTM in CF mainly comprises of *Mycobacterium avium complex* and *M. abscessus*. The increased detection may be partly due to advanced detection and identification of the organisms which are difficult to grow microbiologically [153] and an increased awareness of their prevalence in this population group. Another possibility is the result of antibiotic selection pressure whereby aggressive combinations of antimicrobial treatments may allow individuals who are living longer, to be colonized or infected with environmental organisms which are highly resistant to standard antimicrobials.

It has been shown in recent studies that prophylactic therapy for the dominant pathogen in childhood; *Staphylococcus aureus*, is controversial and there are concerns that treatment may promote earlier infection with *P. aeruginosa* [154, 155].

Prior to the antibiotic era, infants often succumbed to *Staphylococcus aureus* infection but the widespread use of penicillin led to a dramatic improvement in life expectancy. *P. aeruginosa* emerged as a new pathogen in the late 1960s and 1970s [156]. *P. aeruginosa* is extremely problematic as once chronic infection is established, it usually persists for the rest of the patient's lives. It is a major cause of mortality and morbidity [157].

1.2.4 Pseudomonas aeruginosa

It is well established that *P. aeruginosa* is an organism highly adapted to the niche of the airways in CF. Under hypoxic and stressful conditions found in the CF airway, *P. aeruginosa* evolves a range of genetic and phenotypic changes allowing adaptation [158]. In CF, *P. aeruginosa* infection rarely spreads beyond the airways

and mortality is primarily associated with unrelenting airway inflammation and progressive loss of lung function.

The major respiratory pathogen isolated from chronically infected CF patients is a distinctive phenotype of *P. aeruginosa* referred to as 'mucoid' [141]. It is this mucoid strain that is highly adaptive to the CF airway [159]; it lacks invasive virulence factors and instead, produces an alginate capsule [160]. There is an evolutionary shift to increased expression of genes in the algU regulon resulting in downregulation of metabolism, motility and virulence and upregulation of genes controlling efflux and membrane permeability [95]. The hypermutator phenotype results in considerable diversity in the ability to develop antimicrobial resistance; with frequent use of antibiotics being a further driver of resistance (Figures 6 and 7) [161].

The mucoid strain of *P. aeruginosa* has a biofilm mode of growth that allows defence against the innate immune system and antimicrobial therapy. Biofilms are complex extracellular matrix formation and are important factors in *P. aeruginosa* chronic infections [160]. However, non-mucoid strains are better adapted to establish early pulmonary infection and the switch to mucoidity with a downregulation in some virulence factors favours biofilm formation and chronic infection [162]. Studies have shown that aggressive prolonged antimicrobial therapy of non-mucoid strains delayed establishment of chronic infection [159].

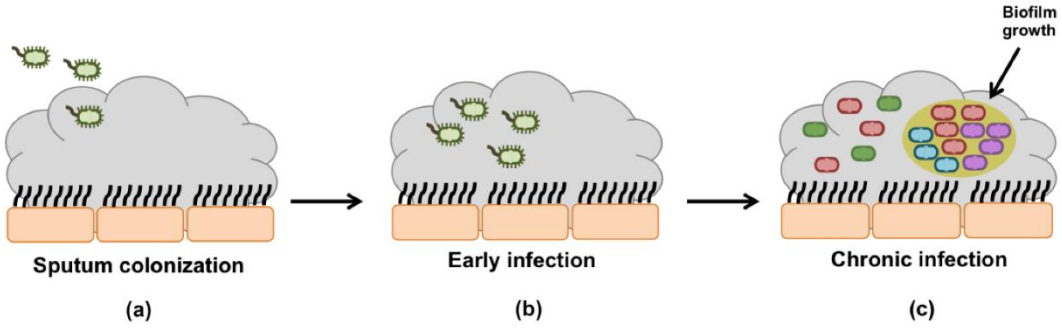

Figure 1-6. Time course of *P. aeruginosa* infection development. (a) Sputum colonization stage - *P. aeruginosa* equipped with full virulence factors enter in CF sputum; (b) Early infection stage—*P. aeruginosa*, which exhibit the environmental or wild-phenotypes species characteristics, starts its adaptation to CF environmental conditions; (c) Chronic infection stage—*P. aeruginosa* is

full adapted to CF environment. At this stage, there is high phenotypic and genotypic diversity and formation of biofilms, image by Sousa *et al*, [161].

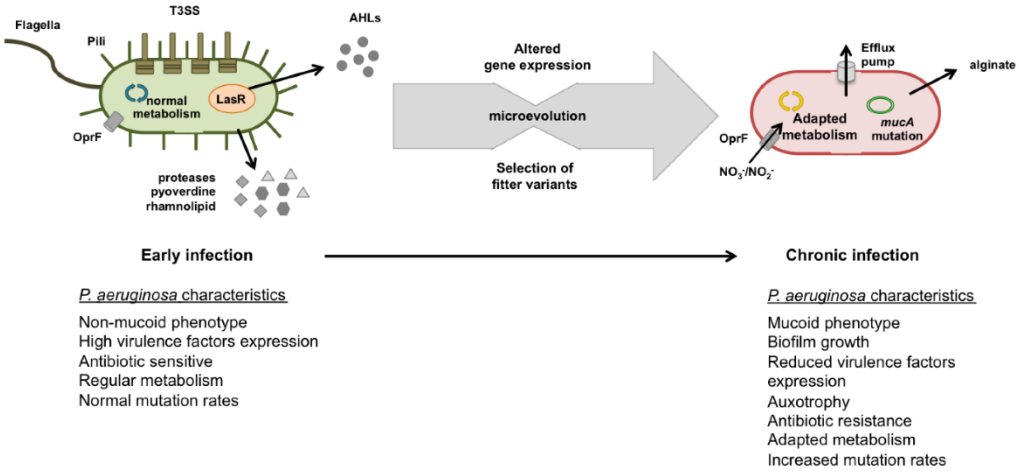

Figure 1-7. Representation of *P. aeruginosa* microevolution during acute to chronic infection in CF airways, image by Sousa *et al*, [161].

Recently, antimicrobial therapy to eradicate initial or repeated episodes *of P. aeruginosa* positive sputum showed a delay in the onset of chronic *P. aeruginosa* which therefore improves life expectancy [162, 163]. The focus here is the ability to 'catch' *P. aeruginosa* infection early enough in order to reduce the likelihood of producing the mucoid strain of *P. aeruginosa* and thus lowering the probability of eradication [164]. This is another reason why taking regular culture of airway samples (surveillance) beginning in infancy, with the aim of detecting early *P. aeruginosa* is vital in improving outcomes in CF [163].

The current route and duration of treatment to eradicate *P. aeruginosa* has not yet been fully established, current practice is using aerosolized antimicrobials such as tobramycin [165]. Indeed, two large studies EPIC [166] and ELITE [167] showed 28 days of aerosolized tobramycin alone led to a negative culture in 90% of patients after 1 month, without the addition of oral antibiotics such as Ciprofloxacin. A later

study utilizing follow up data from the EPIC study showed a sustained *P. aeruginosa* eradication was associated with a 57% reduced risk of the appearance of mucoid *P. aeruginosa* over the follow-up period of 5 years and the median time to first *P. aeruginosa* recurrence was 3.5 years among the sustained eradicators versus only 1 year among the non-sustained eradicators [168].

Eradication presumes that an organism is absent from the airways but to understand this term we also must define chronic infection/ colonisation. There are many definitions that define chronic infection in the CF airways, the most widely used is the Leeds criteria [169]. These criteria classify 'chronic' colonisation when more than 50% of the preceding 12 months were *P. aeruginosa* culture positive, 'intermittent' when < or =50% of the preceding 12 months were *P. aeruginosa* culture positive, 'free of *P. aeruginosa*, with no growth of *P. aeruginosa* for the previous 12 months, having previously been *P. aeruginosa* culture positive, or 'never infected', when *P. aeruginosa* had never been cultured. Validation of this criteria confirmed both a correlation between *P. aeruginosa* status and clinical status as well as with the level of *P. aeruginosa* antibodies with the chronic colonisation group, thus confirming it as a workable classification [170]. A definition of eradication varies widely between clinical trials, although the Cochrane review in 2009 that looked into antibiotic strategies for eradicating *P. aeruginosa* in people with CF, defined eradication as: no isolation of *P. aeruginosa* from bronchoalveolar lavage (BAL), sputum or oropharyngeal cultures at 1, 2, 3, 6, 12 and 24 months after commencement of therapy [171]. It is important to note that eradication often does not mean ‘cure’ and the primary goal of eradication is to delay the onset of chronic colonisation or infection [168]. Failure of eradication often occurs, by means of the recurrence of the initial infecting agent or infection of a new strain of the same bacterial species [172].

It has become apparent using both genomic and phenotypic approaches that populations of *P. aeruginosa* in chronic lung infections are heterogeneous [173, 174]. Isolates from a single sputum sample show considerable phenotypic diversity such as antimicrobial susceptibility and non-mucoid and mucoid strains co-existing.

The communities that exist in the lung micro-environment may therefore have evolved separately [175].

Current microbiological techniques consequently over simplify the complexities of the community by opting to identify a single isolate. For instance, two morphologically identical isolates may give different antibiotic susceptibility results, for the genes and proteins being expressed may be different. This is often why *in vitro* sensitivities do not predict the efficacy of antibiotics in the treatment of chronic infections [176]. Furthermore, resistance to antibiotics is increasing and it is now estimated that 60% of *P. aeruginosa* isolates are classed as multidrug resistant [177]. It is for this reason that *P. aeruginosa* infections are presenting an even greater challenge and health care burden in CF patients.

1.3 The Microbiome in non-CF bronchiectasis and CF

1.3.1 Introduction

Through use of advances in DNA technology, the whole microbial community that exists in the lung environment can now be explored. The most common technique used is 16S rRNA gene sequencing which is a well conserved small subunit of ribsosomal RNA which contains species specific regions that allow PCR amplification through primers [178]. This allows a snapshot view of the overall microbes present as researchers can produce thousands of 16S rRNA sequences per sample by incorporating bar codes in the primer sequences. One can thus assign a frequency to each distinct genome within the community describing either the absolute number of cells in which it is carried or their relative abundance within the population [179].

However, there are some limitations and if the goal of a study is to distinguish species within a genus (e.g. *Staphylococcus aureus* vs *Staphylococcus epidermidis*), longer sequencing reads are often necessary and therefore there is a balancing act between sequence length and sampling depth that must be

considered [178]. Although the 16sRNA gene is highly conserved, several sequenced regions are variable, meaning small numbers of base pairs can change. This means that sequence tags must allow some degree of sequence divergence, typically 95-99% [180]. The resulting cluster of nearly identical sequence tags are referred to as an Operational Taxonomic Unit (OTU) which is the fundamental unit of analysis within a sample [179] (Figure 8). An important concept when dealing with OTUs is that of population diversity [179]. Alpha diversity is the number (richness) and distribution (evenness) of taxa expected within a single population. Additionally, when comparing multiple populations, beta diversity measures the absolute or relative overlap and describes how many taxa are shared between them. An alpha diversity thus acts as a summary statistic of a single population, while a beta diversity acts like a similarity score between populations [179].

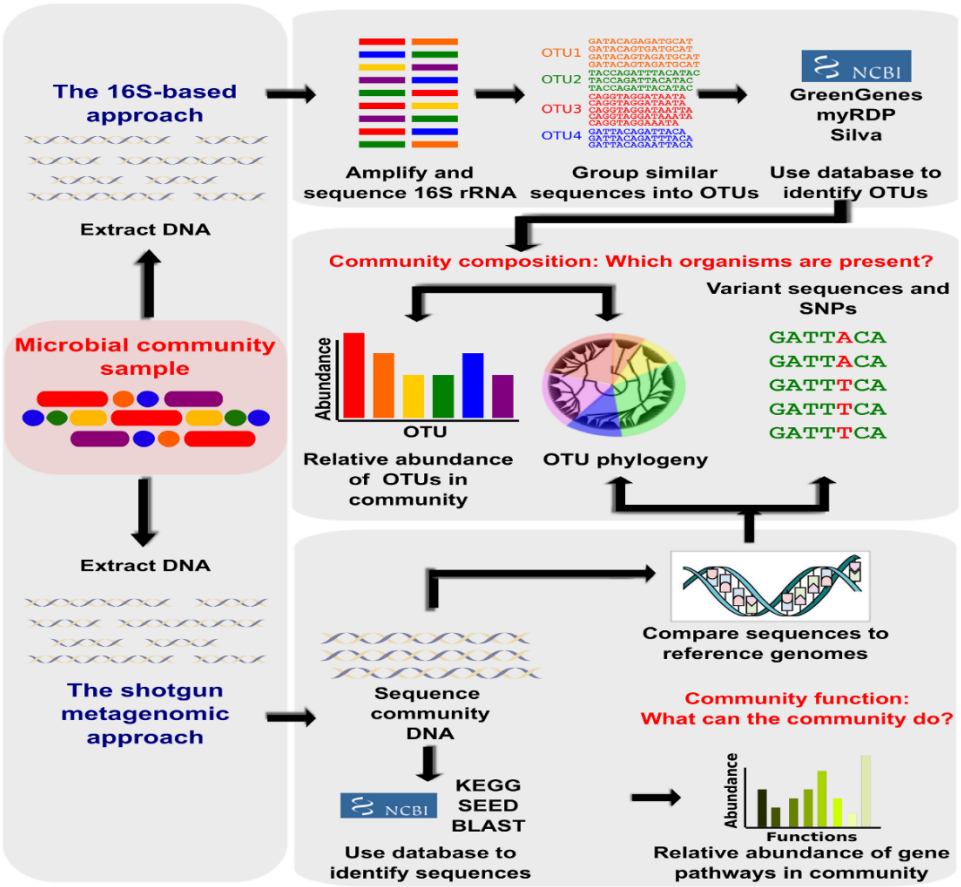

Figure 1-8. The bacterial taxa present in the community are most frequently defined by amplifying and sequencing the 16S rRNA gene. Highly similar sequences are grouped into Operational Taxonomic Units (OTUs), which can be compared to 16S databases. An alternate method of identifying community taxa

is to directly sequence community DNA and compare it to reference genomes. The functional capabilities of the community can also be determined by comparing the sequences to functional databases. This allows the community to be described as relative abundances of its genes and pathways. Image provided by Morgan *et al* [179].

Use of next-generation sequencing has paved the way for whole-genome shotgun (WGS) metagenomics analysis of microbial communities [178]. Metagenomics allows DNA to be extracted directly from a clinical sample and then a library of inserts is constructed by sequencing. This is therefore different from 16S as it explores what microbes are present and answers 'what are they doing'.

Unassembled reads that match signature genes (such as 16S rRNA) can be classified by searching against databases to determine the taxonomic makeup of the metagenomic data set. 16S rRNA read counts are used to calculate the frequency of microbial species, and differences between samples are a measure of relative microbial abundances [178]. Functional data can be inferred from identifying protein-coding genes against reference databases and these are often biased towards organisms that have been already extensively studied [181].

Furthermore, shotgun sequencing has paved the way for metatranscriptomics, which implies shotgun sequencing of reverse-transcribed RNA transcripts [182], metaproteomics is the quantification of protein levels [183] and metametabolomics is the investigation of small-molecule metabolites [184]. One of the greatest obstacles to microbiome analyses through WGS metagenomics is the lack of reference genome sequences. Nevertheless, microbiome studies have shed the light on the diversity of polymicrobial communities existing in the lung environment, which have been overlooked by traditional microbiological culture techniques, and how this may impact on disease.

1.3.2 The non-CF-Bronchiectasis microbiome

Metagenomic studies looking into the non-CF bronchiectasis microbiome have confirmed the airways in bronchiectasis are polymicrobial [185-188]. Notably, anaerobic bacteria were present in high numbers such as *Prevotella, Veillonella* and *Actinomyces* [78]. The data suggests that the microbial community remains relatively stable during periods of exacerbation [78]. This therefore raises the possibility that the function and interactions of the lung microbiota may account for pulmonary exacerbations rather than the composition. It has been suggested in a recent study that a less diverse microbiome dominated by *P. aeruginosa* is associated with a higher future risk of exacerbations and a higher CRP and sputum interleukin-8 [188]. In addition, airway microbiomes rich in *P. aeruginosa,* show a reduced presence of *H. influenzae* [189]. Macrolides used for long-term suppressant therapy, appear to alter the microbiome significantly only in those without *P. aeruginosa* [190]. Rogers *et al* demonstrated a reduction in *H. influenzae* relative abundance with chronic erythromycin administration but interestingly, only those patients with *P. aeruginosa* airway infection, showed a reduced exacerbation frequency with erythromycin [190]. One study also showed that a diverse microbiome was seen in milder disease and therefore correlated positively with lung function and quality of life [187].

Interestingly, studies with comparative data between CF and non-CF bronchiectasis microbiomes using the same analysis techniques are relatively few [191]. Duff *et al* compared the CF and non-CF bronchiectasis microbiota and demonstrated that more than one *Pseudomonas* spp. were present in two patients supporting the observation that both CF and non-CF bronchiectasis can be infected with multiple strains of *P. aeruginosa* [111, 191].

In some studies, researchers have begun to investigate the role of the host response and its relationship to the lung microbiome in various chronic lung diseases [192]. It is now acknowledged that the key to understanding the pathogenesis may reside in deciphering the complex interactions and interplay between host, pathogen and resident microbiota during stability and at exacerbations [193].

1.3.3 The CF microbiome

In the CF lung microbiome, studies have shown that the microbial communities are multispecies and often contain anaerobes [194-196]. In CF, it has been shown that lower species diversity in the sputum microbiome has been associated with worsening lung function [140, 197, 198]. Interestingly, the composition of bacterial communities remains generally stable during exacerbations, despite antibiotic therapy [140, 198-201]. Cross-sectional studies have identified sputum from patients with advanced disease have limited diversity and are dominated by *P. aeruginosa*, *Stenotrophonomas maltophilia* and *Burkholderia cepacia complex* (Bcc) [200, 202].

It is likely, given the complexity of the airway microbiome that microbial interactions play an important role in disease processes. These interactions may be competitive, an example being *P. aeruginosa* attacking other bacteria such as *Staphylococcus aureus* [203-205].

There are a lack of longitudinal studies showing a correlation with CF microbiome and long-term outcomes. However, a recent study did show that decreased alpha diversity of microbial communities in CF airways is predictive of a progression to end stage lung disease and lung function decline [206]. In this study, it showed that sputum microbiota with low alpha diversity dominated with *P. aeruginosa* were more likely to proceed to end stage lung disease or have an exaggerated lung function decline. Conversely, those dominated by organisms such as *Streptococcus*, *Granulicatella* and *Gemella* in a diverse community, were more likely to have a milder clinical course [207]. No other organism was observed in combination with *P. aeruginosa* which appeared to increase the risk of worsening lung decline or end stage lung disease, suggesting that *P. aeruginosa* remains the primary pathogen in CF lung disease [207].

1.3.4 Summary

There continues to be major gaps in our understanding of the evolutionary pressures that promote *P. aeruginosa* persistent infection and what phenotypic

adaptation contributes to worsening patient status both in CF and non-CF bronchiectasis. What is clear is that a lung microbiota dominated by *P. aeruginosa* in CF and non-CF bronchiectasis patients is associated with a worse lung function and outcomes.

A limitation of existing microbiota research in non-CF bronchiectasis and CF is that individual microbes are studied in isolation. Longitudinal studies that link the microbiota to patient's immunology or clinical characteristics coupled with transcriptomic and proteomic data would be more informative [208]. Next generation whole-genome sequencing may offer further insight to this but more work is needed to develop a greater understanding of the different factors which drive disease progression.

1.4 Quorum Sensing molecules as potential biomarkers for infection

1.4.1 Introduction

Bacteria have developed highly sophisticated cell-to-cell communication mechanisms to gather and process environmental information and adapt by regulating the expression of specific genes [209]. It is acknowledged that micro-organisms synthesize, release, detect and respond to small extracellular signalling hormone-like molecules. This process is called 'quorum sensing' (QS), a phenomenon that enables bacteria to sense when the minimum number of cells, or "quorum" is achieved for a concerted response to be initiated [210]. The key components of any QS system are the QS signal synthase, the signal receptor and the signal molecule [211]. The signal molecule then bind with a cell receptor which itself then binds to a promoter region of the signal synthase gene, resulting in a co-ordinated change in gene expression [212]. This results in an autoinduction of signal synthesis and in addition, the signal-receptor complex regulates the expression of a suite of genes, resulting in a change of phenotypic behaviour (Figure 9).

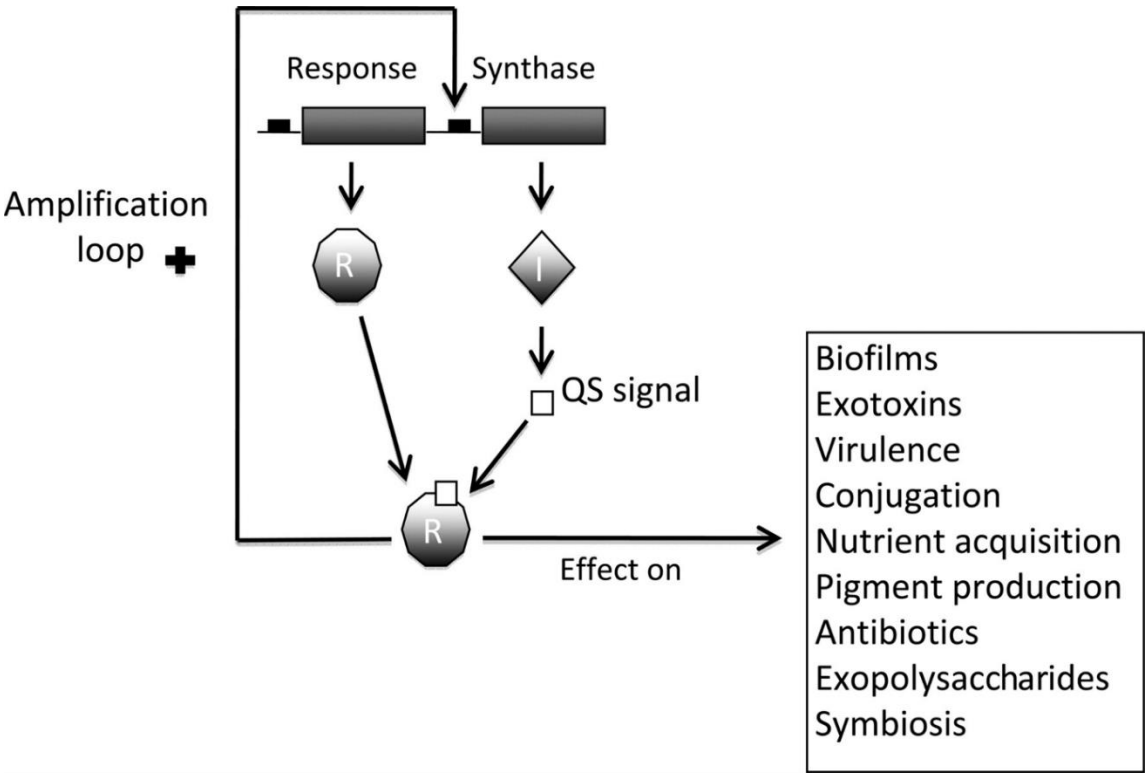

Figure 1-9. . A quorum sensing autoinduction circuit. R represents a receptor/response protein and I represents a QS synthase protein. The binding of signal to the R protein at high cell densities leads to autoinduction of signal synthesis and a change in the behaviour of the bacterial population, image from Popat *et al* [212].

The history of cell-to-cell communication dates back several decades ago. Whilst exploring the marine bioluminescent bacterium *Vibrio fischeri,* which illuminates the light organs of squid, Nealson *et al* noticed that it only occurred at a certain threshold population density of bacterial cells [213]. It was hypothesized that small diffusible molecules that acted as autoinducers, could be sensed by the bacterial cells and this subsequently conveyed information about population density (194). The autoinducer was later identified as N-(3-oxohexanoyl)-homoserine lactone (3-oxo-C5-HSL) which is a member of the N-acyl homoserine lactone (AHL) family of molecules [214]. To date, many bacterial species have been shown to possess QS systems [212]. The signal molecules produced by gram positive bacteria are often peptide derivatives, whereas gram-negative bacteria notably produce AHLs. These are composed of a homoserine lactone ring attached to an acyl side chain via an amide bond [215].

1.4.2 Quorum sensing and virulence

The virulence of an organism will depend upon its ability to produce and release toxins that damage the host. The production of virulence factors requires energy utilisation and therefore it is understandably subject to tightly regulated systems. An effective strategy for bacterial cells to avoid detection and destruction by the host system, is to wait until their numbers are sufficient enough to maximise damage to the host and overwhelm the immune system [212]. QS dependant control of virulence factors has been demonstrated in many bacteria including: *Burkholderia pseudomallei* [216], *Burkholderia mallei* [217], *Burkholderia cepacia* [218], *Vibrio cholera* [219], *Staphylococcus aureus* [220] and lastly, the most widely studied is *P. aeruginosa* [221].

1.4.3 Quorum sensing in *P. aeruginosa*

1.4.3.1 Overview

P. aeruginosa is an extremely environmentally adaptable organism with a large dynamic genome [222], it is estimated approximately 10% of this is devoted to regulatory elements such as the multi-signal QS system [223]. This plays a key role in virulence factor production, expression of antibiotic efflux pumps, swarming motility and biofilm maturation. In addition, the QS molecules involved also directly contribute to host-pathogens interactions [221]. *P. aeruginosa* has four QS systems that function both independently and dependently, namely, the *Las, Rhl*, the quinolone-based QS systems and the recently determined IQS-dependant system [224].

1.4.3.2 N-acylhomoserine lactone-dependant QS in P. aeruginosa

The core of the *P. aeruginosa* QS system consists of two AHL systems, the *lasRI* and *rhlRI* genes, linked to a third circuit. LasR and RhiR are members of the LuxR family of transcriptional regulators that bind to *N*-(3-oxododecanoylhomoserine lactone (3-oxo-C12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL), respectively [225]. In the *las* system, the *LasI* gene promotes the synthesis of 3-oxo-C12-HSL, when the

concentration of this lactone reaches a threshold, it then binds to LasR, which changes gene expression to regulate the production of virulence factors, including elastase, Las A protease, exotoxin A and alkaline protease [226, 227]. In the *rhl* system, the *rhll* gene promotes the synthesis of C4-HSL, which at a threshold level binds to RhIR which changes gene expression and regulates the production of rhamnolipids, pyocyanin, elastase, Las A protease, LecA and LecB lectins [228]. These two systems are organised in a hierarchy in which LasR/3-oxo-C12-HSL drives expression of *Lasl, and rhlR and Rhll,* constituting a positive feedback loop (Figure 10).

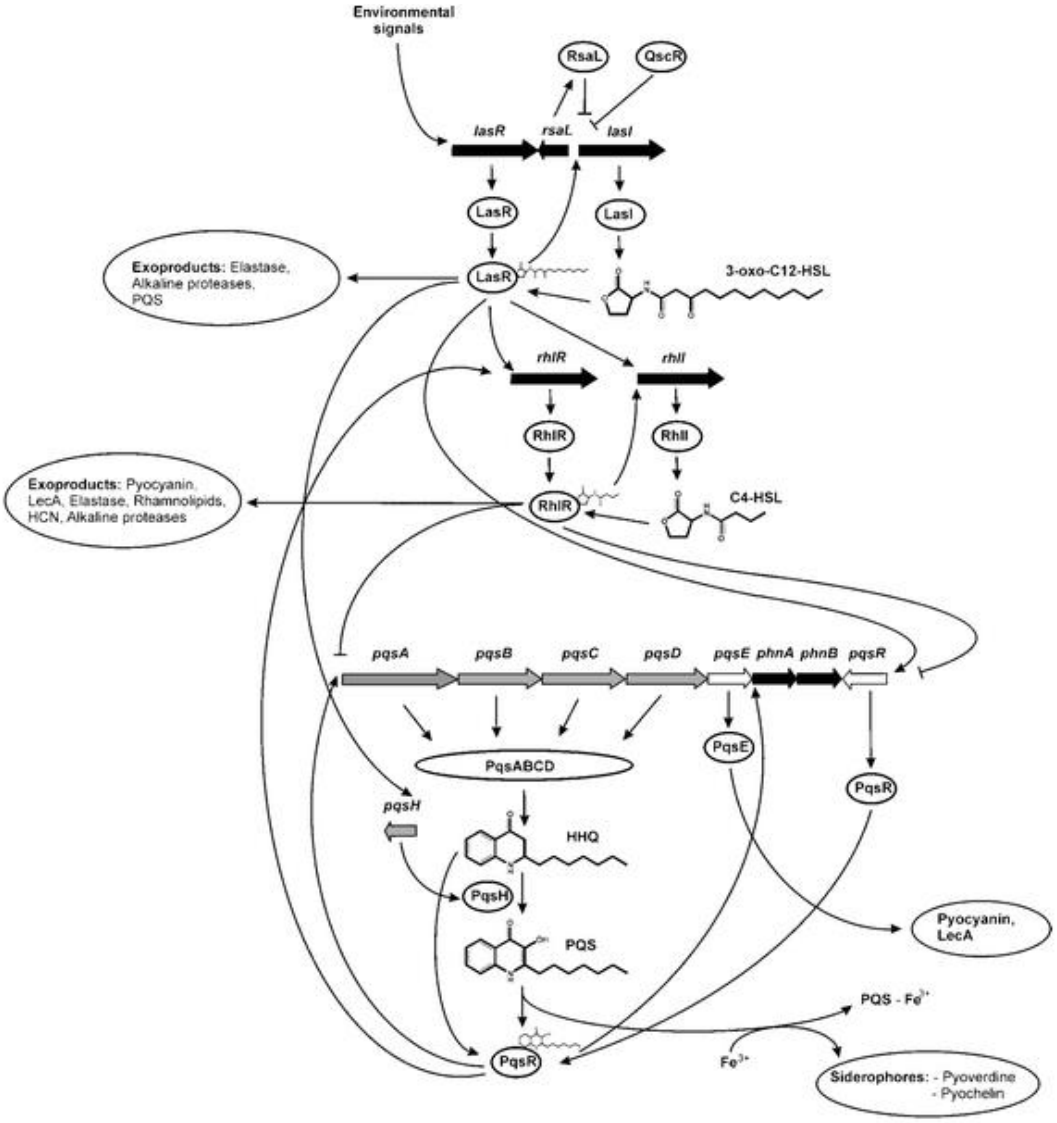

Figure 1-10. Diagrammatic representation of the AHL-dependent and AQ-dependent QS network in *P. aeruginosa* that controls the expression of multiple

virulence determinants. The LasR/3-oxo-C12-HSL complex positively drives the expression of multiple target genes including a second AHL-dependent QS system (RhlRI/C4-HSL) and the AQ-dependent QS system. AHL and AQ-dependent QS are linked since LasR/3-oxo-C12-HSL is required for full expression of pqsH and positively regulates pqsR. Both pqsA and pqsR are repressed by the action of the RhlR/C4-HSL system. ↓ represent positive regulation. ⊥ represent negative regulation, image by Dubern *et al* [229].

In murine models of pulmonary infection with lasl-rhll mutant strains of *P. aeruginosa,* there was an attenuated immune response in the host resulting in a milder lung pathology compared with QS proficient bacteria [230]. In addition, murine models of pneumonia with lasl-rhll mutants, produced thinner and structurally different biofilms compared to wide-type *P. aeruginosa* [231]. Furthermore, Tobramycin, hydrogen peroxide and polymorphonuclear leukocyte activity had a more profound effect on LasR-rhIR mutant biofilms [232].

1.4.3.3 The 2-alkyl-4(1H)-quinolone (AQ) pathway

It was originally demonstrated in 1999 by Pesci *et al* [233] that a non-AHL signal produced by *P. aeruginosa* was responsible for induction of lasB (which codes for las B elastase) in a deficient lasR mutant. The molecule responsible for the non-AHL mediated QS pathway was 2-heptyl-3-hydroxy-4-quinolone and termed Pseudomonas Quinolone Signal (PQS)[234]. PQS belongs to the 2-alkyl-4(1H)-quinolones (AQ) family of compounds, the most biologically active and prevalent are PQS [233], its precursor HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline), C7-PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone), C9-PQS (2-nonyl-3-hydroxy-4(1*H*)-quinolone), HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) and NQNO (2-nonyl-4-hydroxyquinoline-*N*-oxide) [235, 236].

Studies on the synthesis of AQs show that they are derived by the condensation of anthranilate and a β-keto-fatty acid [237]. AQs are produced by *P. aeruginosa* and a few other related bacterial species, including *Burkholderia cepacia* complex and *B.*

pseudomallei [238]. AQs are crucial for bacterial virulence both *in vitro* and in animal models of infection [239]. *P. aeruginosa* produces over 50 of these AQs [235], of which PQS and its precursor HHQ are mostly associated with QS signalling [239]. Some of the AQs have antibiotic [240, 241], immune modulatory [242] and iron-chelating properties [243, 244]. PQS, HHQ and their C-9 congeners are all able to activate PqsR to generate a positive feedback loop. In contrast to HHQ, PQS is an iron chelator and regulates the expression of genes involved in virulence factor production and the iron-starvation response via both PqsR-dependent and PqsR-independent pathways [239, 245].

The production of AQs occurs via a 5-gene operon *pqs*ABCDE, transcription is controlled by a LysR family transcriptional regulator called PqsR [246], which is itself regulated by LasR/3-oxo-C12-HSL [247]. PQS synthesis depends on oxidation of the precursor HHQ by PqsH, which is also LasR dependant [248]. Studies have shown that AQ biosynthesis is positively auto-regulated via PqsR/HHQ/PQS and *las* [247], downregulated by *rhl* and, indirectly, also downregulated via the positive activation of *rhl* [249].

Data from expression studies and virulence factor profiling which was obtained by comparing wild type *P. aeruginosa* strains with *pqs* mutants have revealed the extent of the *pqs* regulon and its relationship with the *las* and *rhl* regulons [239]. It was shown that AQs are required for full transcription of genes coding for exoenzymes, lectins, exotoxins, secondary metabolites such as pyocyanin, rhamnolipids, hydrogen cyanide, pyochelin and pyoverdine and biofilm development [250]. In addition, mutants defective in AQ biosynthesis are severely attenuated in animal and plant infection models [251-253].

1.4.3.4 An integrated system

More recently, a fourth inter-cellular communication signal has been discovered. Lee *et al*, found that this signal was able to integrate environmental stress cues with the quorum sensing network [254]. Named as IQS, it is structurally established as 2-(2-

hydroxyphenyl)-thiazole-4-carbaldehyde and belongs to a new class of QS molecules. The genes that are involved in IQS synthesis are a gene cluster *ambBCDE*. When this gene cluster is disrupted, it caused a decrease in the production of PQS signals well as the virulence factors such as pyocyanin, rhamnolipids and elastase [254]. Furthermore, under phosphate depletion stress conditions, IQS was demonstrated to be able to partially take over the functions of the central *las* system [254].

The recently identified IQS was also found to be tightly controlled by LasR under rich medium conditions. Disruption of either *lasR* or *lasI* completely negated the expression of *ambBCDE* and the production of IQS [254].

1.4.3.5 Bacterial adaptation in chronic infection

It is inevitable that for bacterial pathogens to chronically colonise a host that they must adapt to the host environment and evolve over time within the host [255]. The key drivers for this would be the enhanced survival of the adapted pathogen coupled with the ability to evade the host immune response. Many pathogens persist in the lung due to down regulation of virulence factors and pathogen associated molecular

patterns (PAMPS) to avoid detection by the host (Figure 11).

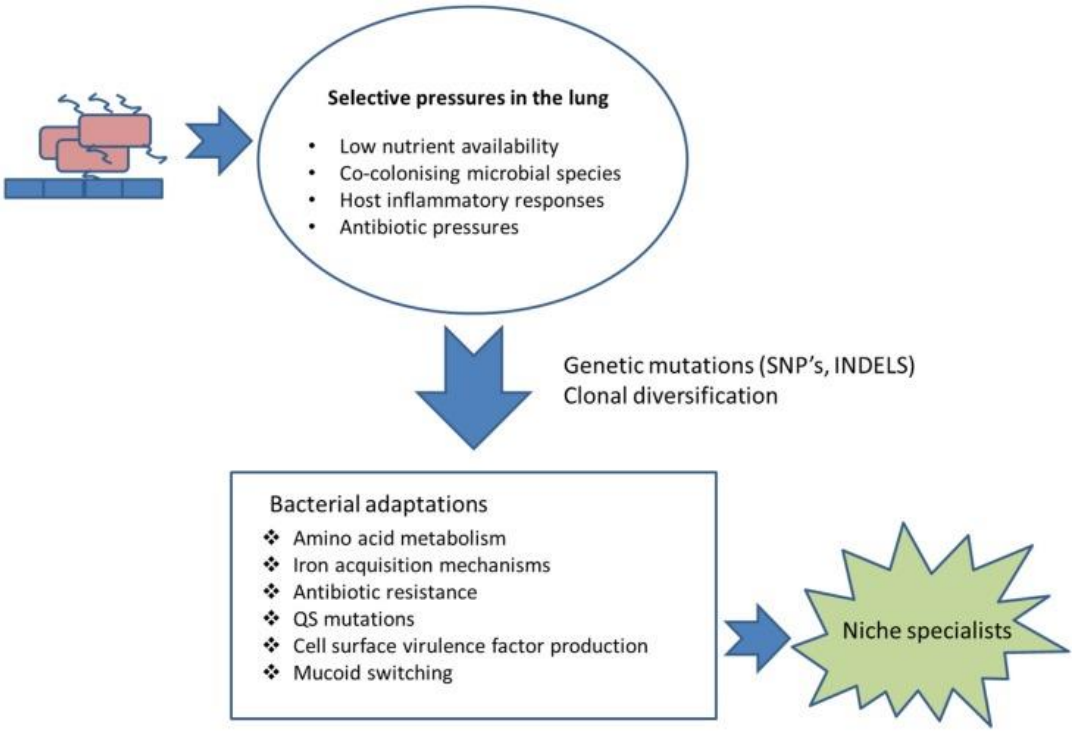

Figure 1-11. Selection and adaptation. Examples of selective pressures to which chronically colonising pathogens are exposed and the adaptations that they undergo, in order to enhance survival, image by Cullen *et al*, [255].

Since virulence factors are generally selected against during chronic infection *in vivo,* and as QS systems control a variety of virulence factor expression, QS mechanisms could be potentially be detrimental to the bacterium during chronic infection [256]. Mutations in LasR have been frequently found in later CF chronic infection. Consequently, these mutants demonstrated a growth advantage with low levels of amino acids and had increased β-lactamase activity *in vitro* [257, 258]. By examining the social behaviour of *P. aeruginosa* in chronic infection*,* Jiricny *et al* showed a statistically significant reduction in the molecules 3-oxo-C12-HSL and PQS, but an increase in HHQ and C4-HSL [259]. This may be explained by the accumulation LasR QS mutants with increasing duration of infection which is a common finding in previous studies [260-262]. This is because the production of HHQ is regulated by the *pqsABCDE* operon and conversion of HHQ into PQS is via PqsH, which is under the control of LasR [229]. It is also known that activity of the *rhl*-system is only

delayed in a *lasR* mutant and both C4-HSL and pyocyanin have been shown to be produced in the absence of LasR [263, 264].

It was thought previously that LasR mutants which are most frequently found in chronic CF lung infections [261], signified presumed inactive QS systems in the lung environment. However, recent data has shown that QS remains active in chronic CF infections and some clinical strains of LasR mutants retain functionality, whilst others had recruited the RhlR-I signalling systems to activate genes [265]. In this same study, both PQS and HHQ were produced in abundance by some LasR-null isolates (245). It has been hypothesized that one possible mechanism for LasR- independent RhlR function is by HHQ or PQS through PqsR induction via an unresolved regulatory mechanism requiring PqsE, an effector protein in the *pqs* system [253, 266, 267]. Interestingly, although LasR mutants are associated with chronic CF infection, there has been no recorded evidence of AQ mutants in this setting.

Another recognised hypothesis for the presence of QS mutants in *P. aeruginosa* chronic lung infections is the exploitation of collective behaviours by 'cheats'. LasR mutants have been shown to act as social cheats in environments where QS is required for growth. This is because cheater cells have a more relative fitness than wildtype strains in mixed populations as they exploit the QS dependant exoproducts produced by wildtype cells [268-270]. This allows the mutants to benefit from the extracellular 'public goods' supply without having to pay the metabolic costs in their production [268].

The interactions of the QS systems which allow *P. aeruginosa* to adapt and persist in the niche of the airways are complex. The QS systems interact closely with one another giving rise to an intricately linked intercellular communication network. This complicated and multi-component QS network enable *P. aeruginosa* to accommodate various environmental cues and biostresses, (Figure 12). In addition, both AHL-dependent and AQ-dependent QS systems are closely linked and mutation of either system results in the attenuation of *P. aeruginosa* virulence.

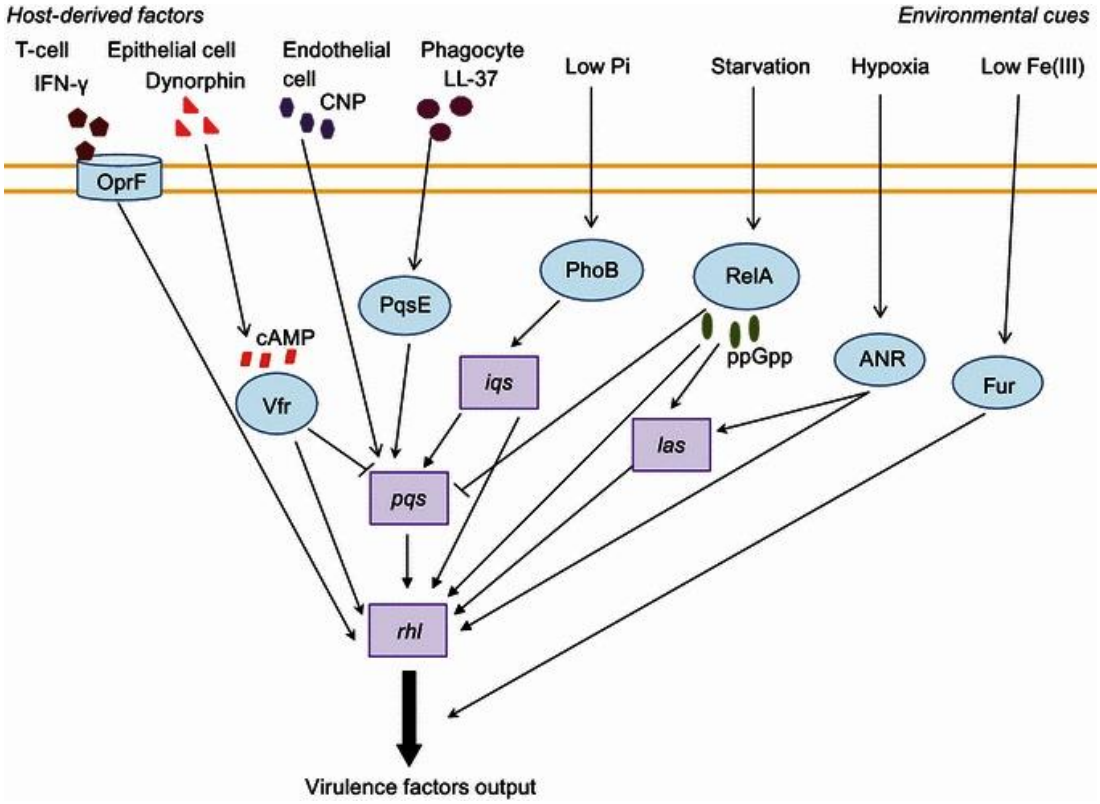

Figure 1-12. Representation of how environmental conditions and host factors influence the QS signalling hierarchy. For simplicity, the QS systems are represented as a whole unit, namely, *las*, *iqs*, *pqs* and *rhl,* image provided by Lee *et al,* [271].

1.4.3.6 Clinical Relevance

The first evidence that AHLs are produced *in vivo* in CF patients was published by The Nottingham quorum sensing group in 2002 [272]. This study used lux-based biosensor strains of *E.coli* to identify AHLs in sputum samples from CF patients infected both with *P. aeruginosa* and *Burkholderia cenocepacia* complex. Furthermore, using liquid chromatography-mass spectrometry, the presence of 3-oxo-C12-HSL in sputum samples from patients colonised with *P. aeruginosa* was confirmed.

This study was closely followed by others which also detected 3-oxo-C12-HSL and C4-HSL in sputum from CF patients [273, 274] and lung tissue [275]. AHLs, namely 3-oxo-C12-HSL, have subsequently been detected in plasma and sputum samples

[276]. 3-oxo-C12-HSL has been detected in the plasma of CF patients and it was shown there was a highly significant correlation found between plasma and sputum measurements of 3-oxo-C12-HSL [277] . A later study in by Struss *et al* showed clinical relevancy of 3-oxo-C12-HSL with links to infection state. Here it was found that 3-oxo-C12-HSL levels in sputum of CF patients were higher in hospitalized patients compared to the levels obtained during their clinically stable periods [278]. There was also noted to be a sudden drop in 3-oxo-C12-HSL concentration from hospital admission to day 2 hospitalisation, thought to be due to the initiation of therapeutic agents [278].

Barr *et al*, reported that AQs are detectable in the sputum, plasma and urine of adults with CF and were positively correlated in all three biofluids [279]. Systemic concentrations of NHQ was elevated at the start of a pulmonary exacerbation and following administration of systemic antibiotics, both concentrations of HHQ and NHQ decreased significantly [279]. Higher systemic concentrations of several AQs were associated with higher *P. aeruginosa* bacterial loads and lower lung function, in cross section analyses [280].

Interestingly, the presence of HHQ in plasma at baseline was significantly associated with a new positive respiratory tract *P. aeruginosa* culture in the following year, showing its potential as an early biomarker of infection [280].

HHQ's role *in vitro* with transcriptome data is that it purely acts as an autoinducer generating a positive feedback loop that accelerates AQ biosynthesis [239] . Microarray data has showed that, in contrast to HHQ, PQS regulates the expression of 182 genes. Genes involved include promoting production of the iron-chelating siderophores; pyoverdine and pyochelin, [245, 281] which confer a survival advantage to *P. aeruginosa* in mixed bacterial populations [282]. In addition, PQS has been shown to upregulate numerous key virulence genes including proteases, fumarate hydratase, superoxide dismutase, exotoxins and the Type 3 secretion system[239]. PQS has also been shown to regulate the production of elastase, pyocyanin. cyanide and LecA lectin and also play a role in regulating antibiotic

resistance and biofilm maturation [246, 263, 283]. Pyocyanin is known to be a key virulence factor that plays a major role in CF patients [284].

1.4.3.7 QS inhibitors as drug targets for P. aeruginosa

In vitro studies have shown that macrolides interfere with *P. aeruginosa* QS pathways [285-287]. Hoffman *et al,* found in a murine model of infection that Azithromycin specifically blocked LasB dependant QS which resulted in reduced alginate production and increased bacterial clearance of *P. aeruginosa* [288]. On a similar note, Nagata *et al* showed that erythromycin suppresses biofilm formation in murine models of infection [289]. A recent study showed that despite erythromycin therapy having no significant impact on *P. aeruginosa* bacterial load in sputum *in vivo*, it did change *P. aeruginosa* gene expression of both LasR and *pqsA* in non-CF bronchiectasis patients [127]. In addition, Skindersoe *et al* found that ciprofloxacin and ceftazidime as well as macrolides can reduce QS dependant production of virulence factors in laboratory strains of *P. aeruginosa* [290].

In recent years, attempts to develop new classes of antimicrobial agents have included the targeting of virulence factors or virulence regulatory mechanisms. One such strategy could be directed toward interference with QS-mediated signalling to disrupt bacterial communication in order to attenuate virulence [291]. It is proposed that QS inhibitors (QSI) would unlikely be sufficient to eradicate an infection on their own, but may act synergistically in combination with antibiotics. The use of QSI would not compromise bacterial viability and therefore should theoretically impose a lesser selection pressure to drive bacterial resistance compared to antibiotics [292].

Recent work has confirmed that pharmacological inhibition of the LasR receptor induces and stabilizes conformational changes that prevent the complex (LasR-antagonist) from binding to DNA and thereby preventing transcription of the target genes [293]. The *las* system has been the most extensively investigated as an anti-virulence drug target. Despite the numerous attempts to target signal synthesis (Lasl) or signal reception (LasR), most of these inhibitors have lacked the lead-like

properties required and have failed to proceed to clinical development due to drawbacks such as lack of uniformity and standardization [291]. It is also worth noting that LasR mutants, as previously discussed, frequently arise in chronic human *P. aeruginosa* infections [265]. With respect to *rhl* inhibition, it is yet not clear whether inhibiting this system alone would yield therapeutic benefits, furthermore, there is a lack of structural information for RhlR and Rhll [294].

The *pqs* system in *P. aeruginosa* is crucial for virulence and persistence in the human host, it also has immune modulatory effects [295]. Now that the molecular and structural basis of the *pqs* biosynthesis and signal transduction pathways have been elucidated, it represents an attractive drug discovery target.

Recent work exploring *pqs* inhibitors has showed promising results leading to disruption of biofilm formation and pyocyanin production [296]. Interestingly a recent study using compounds that inhibit both *las, rhl* and *pqs* systems has been shown to down regulate multiple virulence factors such as pyocyanin, rhamnolipids and elastase and exhibited a high clearance rate of bacteria in mice [297].

In summary, *P. aeruginosa* is intrinsically resistant to many antibiotics and possesses a great challenge to patients who are chronically colonised such as those in non-CF bronchiectasis and CF. With anti-microbial resistance imposing a global health threat, there is an urgent need to explore new classes of antibacterial agents with novel modes of action, such as anti-virulence therapy.

1.5 Hypothesis and aims of this thesis

1.5.1 Project aims

Persistent bacterial infection of the airway mucosa in both non-CF bronchiectasis and CF patients are a key driving event for the complex pathological processes that are involved in these conditions. Pulmonary exacerbations and chronic infection are a huge healthcare burden in both patient cohorts.

It is clear that *P. aeruginosa* causes a greater mortality, morbidity and reduced quality of life in both bronchiectasis and CF. Nevertheless, CF has been subject to more research in this area in previous years. In particular, the heterogeneity of conditions in bronchiectasis need a greater understanding both into drivers of pathogenicity and the appropriate diagnostic and management strategies.

1.5.2 Overall aims of this thesis:

1. To explore the potential of QS molecules as biomarkers of disease progression and long term adverse outcomes in people with CF.
2. To explore the anaerobic microbiota in people with CF chronically colonised with *P .aeruginosa* and the association with lung function decline.
3. To establish a feasibility study in non-CF bronchiectasis to explore QS molecules in this cohort. In addition, to determine both clinical and microbiological factors that may influence disease severity, time between exacerbations and mortality.
4. To explore urinary biomarkers of clinical status in CF and non-CF bronchiectasis that may predict pulmonary exacerbations or treatment response.
5. To investigate non-invasive biomarkers of *P. aeruginosa* pulmonary infection that may be predictive of early or chronic infection in CF and non-CF bronchiectasis.

Chapter 2. Clinical significance of *Pseudomonas aeruginosa* 2-alkyl-4-quinolone quorum sensing signal molecules for long-term outcomes in adults with cystic fibrosis

2.1 Introduction

This chapter describes a prospective cohort study which explored the association of 2-alkyl-4-quinolones (AQ) quorum sensing signal molecules for *P. aeruginosa* measured in sputum or blood with long term adverse clinical outcomes in adults with cystic fibrosis.

2.1.1 Background

P. aeruginosa is a highly successful opportunistic gram-negative bacterium which is well adapted to the airway niche in cystic fibrosis (CF). *P. aeruginosa* is the dominant pathogen in the CF lung and is associated with increased morbidity and mortality in this population [157]. *P. aeruginosa* is intrinsically resistant to many classes of antibiotics, produces a host of virulence factors and forms impenetrable biofilms in the CF airways [298]. *P. aeruginosa* controls the production of these virulence factors using a cell-to-cell communication known as quorum sensing (QS) [299]. This allows the whole bacterial population to sense and respond to changes in environmental stimuli and to coordinate gene expression of the community as a whole.

The *P. aeruginosa* QS system consists of 3 interlinking QS circuits, one of which is the *pqs* QS circuit. The *pqs* QS systems uses multiple 2-alkyl-4 quinolones (AQs) as signal molecules, including the pseudomonas quinolone signal (PQS) molecule (2-heptyl-3-hydroxy-4(1*H*)-quinolone) and its precursor HHQ (2-heptyl-4-

hydroxyquinoline) [233]. Both PQS and HHQ act as autoinducers to increase AQ biosynthesis [239] . In addition, PQS is crucial for the production of virulence factors and biofilm formation both *in vitro* and in animal models of infection [239]. *P. aeruginosa* produces over 50 AQs, and the roles of many of these AQs are not yet fully understood [235].

Several AQs are detectable in the sputum and plasma of adults with CF and chronic pulmonary *P. aeruginosa*. Higher systemic concentrations of several AQs are associated with higher *P. aeruginosa* loads and lower lung function, in cross section analyses [280, 300]. This suggests that high systemic AQ levels may be associated with an adverse prognosis. In addition, systemic concentrations of several AQs decrease following intravenous anti-pseudomonal antibiotics, [300] suggesting they have potential as biomarkers of change in clinical status.

2.1.2 Aims

We hypothesised that higher levels of AQs in the sputum and plasma of adults with CF would be associated with mortality, lung function decline and antibiotic use in this patient population.

We investigated whether detection of six individual AQs in the sputum and plasma of people with CF could be linked to long-term outcomes, including death or lung transplantation, as well as annual rate of lung function decline and intravenous (IV) antibiotic use for pulmonary exacerbations. Clinical data over an 8 year period was retrospectively collected on 90 adults with CF who had previously participated in an AQ biomarker study [280].

2.2 Methods

2.2.1 Participants

We studied 90 adults with CF who had previously participated in an AQ biomarker study, the full details of which were previously published [280]. In summary, participants were recruited at clinical stability from two UK adult CF centres between the years 2009 and 2011. Inclusion criteria were a diagnosis of CF and exclusion criteria were any previous isolation of *Burkholderia cepacia complex* in sputum. Baseline demographic data and data on six AQs measured in both sputum and plasma samples were used [280].

2.2.2 Study design

Follow up clinical data were retrospectively obtained from the UK CF registry. Annual data were collected from the participants from the year of recruitment to the end of the study period in 2017. Data on death, lung transplantation, lung function and the number of IV antibiotic days for pulmonary exacerbations were obtained. The number of IV antibiotic days was measured annually from the year of recruitment to the end of 2017. For lung function data, the best recorded forced expiratory volume in 1 s (FEV_1) of the preceding year was used.

The primary outcomes were death or lung transplantation during the follow up period. Secondary outcomes were the number of IV antibiotic days for pulmonary exacerbations and rate of decline in FEV_1.

2.2.3 Sample processing and AQ analysis

All sample processing and AQ analyses were performed in the initial study as previously described [280]. Sputum plugs were harvested for quantitative AQ analyses [301, 302]. Venous blood samples were centrifuged at 1000 *g* for 15 min at 4°C, plasma was then separated and snap frozen in liquid nitrogen. Sputum samples for AQ analysis were extracted using acidified ethyl acetate (Fisher Chemicals, Loughborough, UK)

[301, 302]. Plasma samples were extracted by solid phase extraction and plasma matrix matched samples from a healthy volunteer donor were prepared to allow calibration of samples. Prepared clinical samples for AQ analyses were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Ortori *et al* 2011 [301]. All samples were analysed once using LC-MS/MS with no replicate analysis performed.

The lower limit of quantification (LLOQ) was established by using serial dilutions of the analyte mix and spiking into blank plasma samples prior to extraction and analysis, The LLOQ for plasma was defined as the analyte concentration at which a signal/noise ratio of 10:1 was achieved. In the absence of blank sputa to produce matrix matched calibration, 1.0mL aliquots of 0.9% NaCl were used and there was no LLOQ defined for sputum samples. A total of six AQs were analysed individually: HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline), PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone), C9-PQS (2-nonyl-3-hydroxy-4(1*H*)-quinolone), HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) and NQNO (2-nonyl-4-hydroxyquinoline-*N*-oxide). Calculated LLOQs in plasma samples were as follows: HHQ 10 pmol/L; NHQ 10 pmol/L; HQNO 30 pmol/L, NQNO 40 pmol/L; PQS 100 pmol/L and C9-PQS 100 pmol/L [280]. Quantitative concentrations for the six AQs both in plasma and sputum are summarised in Table 2-1.

Table 2-1. Individual AQ concentrations measured at baseline clinical stability from sputum and plasma.

AQ	Sputum pmol/L :median (range)	Plasma pmol/L: median (range)
HHQ	18 (0 to 417)	61 (0 to 867)
NHQ	16 (0 to 625)	0 (0 to 139)
PQS	2 (0 to 431)	0 (0 to 458)
C9-PQS	17.5 (0 to 417)	0 (0 to 40)
HQNO	17.5 (0 to 1318)	0 (0 to 879)
NQNO	20 (0 to 858)	0 (0 to 853)
0: concentration below the lower limit of quantification; AQ: 2-alkyl-4 quinolones,HHQ; 2-heptyl-4-hydroxyquinoline, NHQ; 2-nonyl-4-hydroxyquinoline; PQS; 2-heptyl-3-hydroxy-4(1*H*)-quinolone, C9-PQS; 2-nonyl-3-hydroxy-4(1*H*)-quinolone, HQNO; 2-heptyl-4-hydroxyquinoline-*N*-oxide, NQNO; 2-nonyl-4-hydroxyquinoline-*N*-oxide.		

2.2.4 Statistical Analysis

The six measured AQs were analysed individually and combined detectable AQ levels in sputum and plasma were calculated.

For initial analyses, individual AQ levels were classified as detectable or undetectable (concentrations above or below the LLOQ respectively). A binary measure of combined AQs was defined as a detection of at least one individual AQ in sputum or plasma respectively. Two-sample Wilcoxon rank-sum (Mann-Whitney) tests and Pearson's chi-squared tests were used to assess binary AQ levels with the primary and secondary clinical outcomes. Kaplan-meier plots were generated to visually represent survivor probability (the absence of death or lung transplantation) with total combined sputum and plasma AQs over the study period (censored to 2017)

If significant associations were obtained using binary AQ analyses, further quantitative analyses were performed. Individual AQ concentrations were then compared with clinical outcomes using Spearman rank correlation coefficients. Statistical significance

was assessed as $p<0.05$. All data were analysed using Stata SE15 statistical software (Texas, USA).

2.3 Results

Of the 90 participants in the original study, 7 were lost during follow up and therefore 83 participants were included in the analyses. Baseline characteristics are summarised in Table 2-2.

The median follow up period was 6.3 years (IQR 5.6 to 6.7 years). During the follow up period, 23 participants (27%) either died or had bilateral lung transplantation. Three people had a bilateral lung transplant and subsequently died during the follow up period. These characteristics are summarised in Table 2-3.

Table 2-2. Baseline clinical characteristics and *P. aeruginosa* status of participants.

Variable	Baseline (n=83)
Nottingham University Hospitals NHS trust	42
University Hospitals Birmingham NHS Foundation Trust	41
Age in years: median (range)	28.4 (17.8 to 61.5)
Gender, males (%)	54 (65.1)
FEV_1 % predicted: mean (SD)	58 (±20)
Absolute FEV_1 in L: mean (SD)	2.13 (±0.9)
BMI: mean (SD)	22.9 (± 3.3)
P. aeruginosa status at baseline: n (%)	
Never	0 (0)
Free	1 (1.2)
Intermittent	2 (2.4)
Chronic	80 (96.4)

n = number of participants with data available; SD = standard deviation
P. aeruginosa status of participants defined by Leeds criteria [169]

Table 2-3. Summary of clinical data during follow up period.

Variable	N	(%)	Outcome
Follow up time¥	83	100.0%	6.3 (5.6-6.7)
Number of deaths/lung transplantation	23	27.7%	
Died during follow up	15	18.1%	
Rate of decline per year§:			
Absolute FEV_1 (ml)	80	96.4%	53.1 (55.5)
Percent predicted FEV_1 (%)	80	96.4%	1.6 (2.0)
Number of IV antibiotic days per year¥:			
Overall	81	97.6%	37.5 (16.4-58.7)
No death/transplant	59	71.1%	31.9 (13.0-43.7)
Death/transplant*	22	26.5%	60.1 (46.5-80.4)

¥: reported as median and interquartile range. § reported as mean and standard deviation. *Mann-Whitney significance p<0.001. IV= intravenous. N= number of participants with data available.

2.3.1 Presence or absence of detectable levels of AQs at baseline on primary outcomes

Death or lung transplantation during follow up was not statistically different in the presence or absence of detectable levels of six individual AQs at baseline (Table 4), using binary AQ analyses (detected versus not detected). Similarly, there were no statistical associations between the combined AQs measured and primary outcomes, indicating independence (chi-squared p=0.751 and p= 0.351 for total sputum and plasma AQs respectively, Table 2-4).

Table 2-4. Individual and combined total AQs with primary outcome of death or lung transplantation.

AQ	Death/transplant: n(%)	No Death/transplant: n(%)	*p*-value (chi²)
Sp HHQ +	14 (70)	34 (65.4)	0.71
Sp HHQ -	6 (30)	18 (34.6)	
Pl HHQ +	14 (66.7)	35 (58.3)	0.501
Pl HHQ -	7 (33.3)	25 (41.7)	
Sp NHQ +	15 (75)	35 (67.3)	0.526
Sp NHQ -	5 (25)	5 (32.7)	
Pl NHQ +	3 (14.3)	18 (30)	0.157
Pl NHQ -	18 (85.7)	42 (70)	
Sp PQS +	12 (60)	31 (59.6)	0.98
Sp PQS -	8 (40)	21 (40.4)	
Pl PQS +	8 (38.1)	20 (33.3)	0.693
Pl PQS -	13 (61.9)	40 (66.7)	
Sp C9-PQS +	15 (75)	33 (63.5)	0.352
Sp C9-PQS -	5 (25)	19 (36.5)	
Pl C9-PQS +	3 (14.3)	5 (8.3)	0.431
Pl C9-PQS -	18 (85.7)	55 (91.7)	
Sp HQNO +	16 (80)	39 (75)	0.655
Sp HQNO -	4 (20)	13 (25)	
Pl HQNO +	11 (52.4)	28 (46.7)	0.652
Pl HQNO -	10 (47.6)	32 (53.3)	
Sp NQNO +	14 (70)	39 (75)	0.666
Sp NQNO -	6 (30)	13 (25)	
Pl NQNO +	8 (38.1)	21 (35)	0.799
Pl NQNO -	13 (61.9)	39 (65)	
Total Sp AQ+	18 (90)	48 (92.3)	0.751
Total Sp AQ -	2 (10)	4 (7.7)	
Total Pl AQ +	15 (71.4)	36 (60)	0.351
Total Pl AQ -	6 (28.6)	24 (40)	

+ detected; - undetected; Sp=Sputum; Pl=Plasma; AQ=2-alkyl-4 quinolones; n= number; HHQ= 2-heptyl-4-hydroxyquinoline; NHQ= 2-nonyl-4-hydroxyquinoline; PQS=2-heptyl-3-hydroxy-4(1*H*)-quinolone; C9-PQS= 2-nonyl-3-hydroxy-4(1*H*)-quinolone; HQNO= 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO= 2-nonyl-4-hydroxyquinoline-*N*-oxide

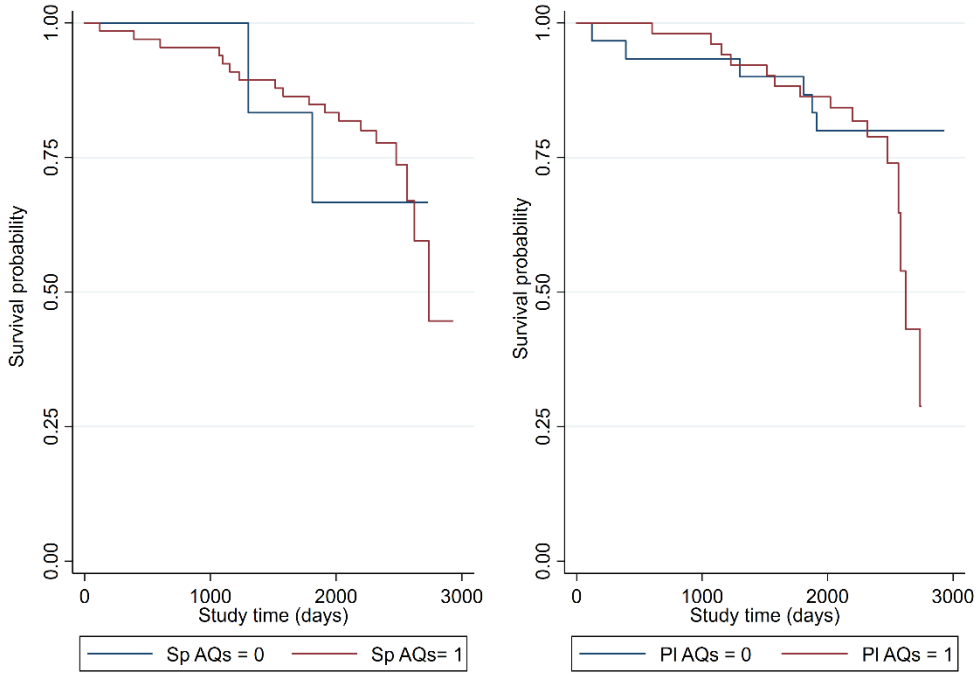

Figure 2-1. Kaplan-Meier survival estimates on death or lung transplantation with total combined sputum and plasma AQs.

Figure2-1 Legend. Kaplan-Meier survival estimates graph comparing time to death or lung transplantation in the study period with total combined sputum and plasma AQs. Sp; sputum, Pl; Plasma, AQ;2-alkyl-4 quinolones Survival probability; in the absence of death or lung transplantation.

2.3.2 The presence or absence of detectable levels of AQs at baseline on secondary outcomes

There was no statistical difference demonstrated with rate of FEV_1 decline both with individual and combined AQs detected in plasma or sputum (Table 2-5).

There was no association between five of the individual AQs and number of IV antibiotic days per year (Table 6).

The presence of C9-PQS in the sputum was associated with an increase in IV antibiotic days per year during the follow up period (Mann-Whitney p=0.011; Figure 2-2). The median number of IV antibiotic days per year if sputum C9-PQS was detected was 41.4 (IQR: 26.6 to 60.7) compared with 28.2 (IQR: 4.0 to 44.4) when not detected. A similar

finding was observed when follow up was restricted to 3 years; 44.8 (IQR: 25.1 to 62.1) when sputum C9-PQS was detected compared with 29.7 (IQR: 4.7 to 50.4) when not detected (Mann-Whitney p=0.046). The concentration of C9-PQS in sputum was positively correlated with number of IV antibiotics per year but did not reach statistical significance (Spearman rank correlation; r= 0.2, p= 0.09; Figure 2-3). There was no statistical difference in the number of IV antibiotic days when C9-PQS was detected in plasma (Table 2-6; Mann-Whitney p=0.32), nor detectable levels of total combined AQs in plasma or sputum (Table 2-6). The number of IV antibiotic days per year was statistically higher in people who died or had a bilateral lung transplantation in the follow up period (Mann-Whitney p<0.001, Table 2-3).

Table 2-5. Individual and combined AQs with of rate of FEV_1 decline.

AQ (n)	Rate FEV_1 ml/yr: mean (SD)	p- value*
Sp HHQ + (n=47)	48.2 (± 62.1)	0.403
Sp HHQ - (n=23)	63 (± 82.6)	
Pl HHQ + (n=49)	48.3 (±62.7)	0.289
Pl HHQ - (n=30)	64.6 (±70.8)	
Sp NHQ + (n=49)	53.8 (±70.8)	0.887
Sp NHQ - (n=21)	51.2 (±67.2)	
Pl NHQ + (n=21)	31.1 (±55.6)	0.057
Pl NHQ - (n=58)	62.9 (±67.7)	
Sp PQS + (n=42)	44.1 (±59.5)	0.186
Sp PQS - (n=28)	66.5 (±81)	
Pl PQS + (n=28)	48 (±67.2)	0.519
Pl PQS - (n=51)	58.1 (65.6)	
Sp C9-PQS + (n=47)	42.6 (±65.6)	0.071
Sp C9-PQS - (n=23)	74.4 (±76.6)	
Pl C9-PQS + (n=8)	22.7 (±57.8)	0.152
Pl C9-PQS - (n=71)	58.1 (±66.2)	
Sp HQNO + (n=54)	53.5 (±68.4)	0.926
Sp HQNO - (n=16)	51.6 (±74.3)	
Pl HQNO + (n=39)	51.3 (±66.1)	0.674
Pl HQNO - (n=40)	57.6 (±66.5)	
Sp NQNO + (n=52)	51.9 (±69.2)	0.815
Sp NQNO - (n=18)	56.4 (±71.1)	
Pl NQNO + (n= 29)	46.2 (±62.1)	0.398
Pl NQNO - (n=50)	59.3 (±68.2)	
Total Sp AQ + (n=64)	50.7 (±64.7)	0.367
Total Sp AQ - (n=6)	77.6 (±112.1)	
Total Pl AQ + (n=51)	48.1 (±61.5)	0.247
Total Pl AQ - (n=28)	66.1 (±73.1)	

+ detected; - undetected; Sp=Sputum; Pl=Plasma; AQ=2-alkyl-4 quinolones; n= number; HHQ= 2-heptyl-4-hydroxyquinoline; NHQ= 2-nonyl-4-hydroxyquinoline; PQS=2-heptyl-3-hydroxy-4(1*H*)-quinolone; C9-PQS= 2-nonyl-3-hydroxy-4(1*H*)-quinolone; HQNO= 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO= 2-nonyl-4-hydroxyquinoline-*N*-oxide, n; number of observations; SD; standard deviation; *; p-value determined by paired ttest

Table 2-6. Individual and combined AQs with number of IV antibiotic days a year.

AQ(n)	IV antibiotic d/yr: median (IQR)	p-value*
Sp HHQ + (n=48)	38.1 (20.3 to 58.8)	0.504
Sp HHQ - (n=24)	37.7 (13 to 56.5)	
Pl HHQ + (n=49)	37.5 (14.4 to 58.7)	0.622
Pl HHQ - (n=32)	37.3 (17.3 to 54.9)	
Sp NHQ + (n=50)	38.1 (24 to 57.6)	0.513
Sp NHQ - (n=22)	37.7 (13 to 61.3)	
Pl NHQ + (n=21)	36.9 (24 to 68.9)	0.535
Pl NHQ - (n=60)	37.5 (15.4 to 57.1)	
Sp PQS + (n=43)	38.8 (25.6 to 58.7)	0.143
Sp PQS - (n=29)	34.6 (4.8 to 57.6)	
Pl PQS + (n=28)	39.2 (18.2 to 57.1)	0.827
Pl PQS - (n=53)	36.9 (16.4 to 58.7)	
Sp C9-PQS + (n=48)	41.4 (26.6 to 60.7)	0.011
Sp C9-PQS - (n=24)	28.2 (4 to 44.4)	
Pl C9-PQS + (n=8)	44.9 (30.4 to 67.9)	0.326
Pl C9-PQS - (n=73)	36.9 (14.4 to 56.5)	
Sp HQNO + (n=55)	37.7 (14.4 to 58.7)	0.686
Sp HQNO - (n=17)	37.6 (16.6 to 55.3)	
Pl HQNO + (n=39)	38.8 (24 to 63.8)	0.369
Pl HQNO - (n=42)	36.5 (13 to 55.3)	
Sp NQNO + (n=53)	37.5 (14.4 to 56.5)	0.808
Sp NQNO - (n=19)	38 (16.6 to 64.2)	
Pl NQNO + (n=29)	37.5 (24 to 57.6)	0.790
Pl NQNO - (n=52)	37.3 (15.4 to 57.6)	
Total Sp AQ + (n=66)	37.9 (16.6 to 57.6)	0.839
Total Sp AQ - (n=6)	36.2 (3.8 to 77)	
Total Pl AQ + (n=51)	37.5 (13.7 to 62.5)	0.646
Total Pl AQ - (n=30)	37.3 (18 to 54.5)	

+ detected; - undetected; Sp=Sputum; Pl=Plasma; AQ=2-alkyl-4 quinolones; n= number; HHQ= 2-heptyl-4-hydroxyquinoline; NHQ= 2-nonyl-4-hydroxyquinoline; PQS=2-heptyl-3-hydroxy-4(1*H*)-quinolone; C9-PQS= 2-nonyl-3-hydroxy-4(1*H*)-quinolone; HQNO= 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO= 2-nonyl-4-hydroxyquinoline-*N*-oxide, n; number of observations, IQR; interquartile range; d/yr; days per year; *; p-value determined by Mann-Whitney test.

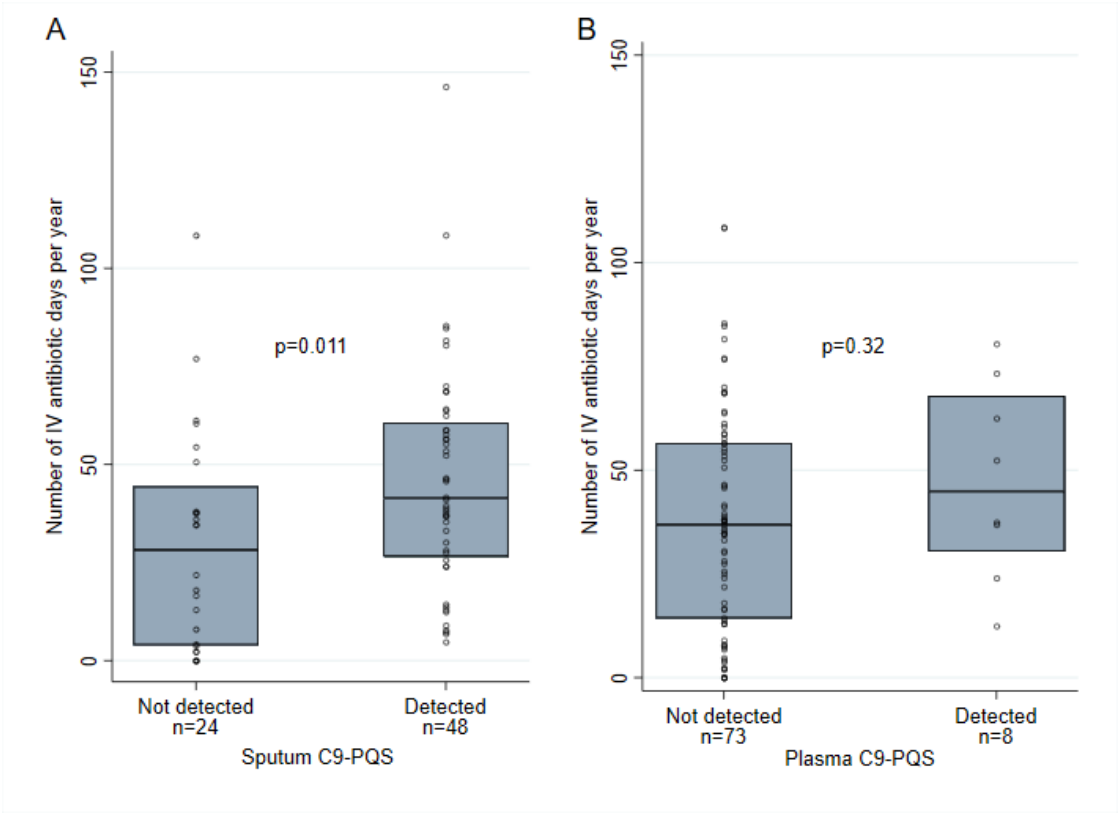

Figure 2-22. Annual number of IV antibiotic days according to C9-PQS detection at baseline.

Figure 2-2 legend: Box plot showing the relationship between the presence and absence of detectable levels of sputum and plasma C9-PQS at baseline with the number of intravenous antibiotics days per year during the follow up period. Box represents interquartile range, Line represents median value. C9-PQS= 2-nonyl-3-hydroxy-4(1H)-quinolone; IV= Intravenous; p value derived from Mann-Whitney test; n= number of observations.

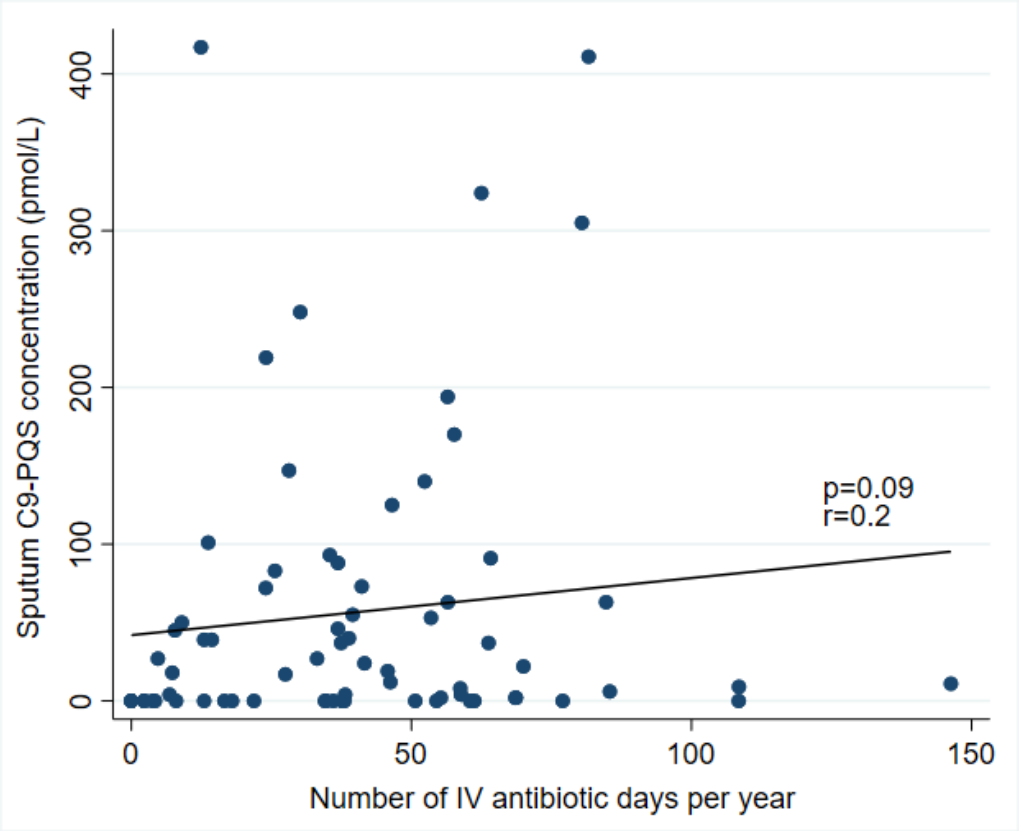

Figure 2-3. Scatter graph of number of IV antibiotic days per year with sputum C9-PQS concentration (pmol/L).

2.4 Discussion

This is the first study to explore the relationship between baseline AQ quorum sensing signal molecules measured in sputum or plasma with long term outcomes in adults with CF. There were no significant difference between the detection of sputum or plasma AQs at baseline with death, lung transplantation or rate of FEV_1 decline over the follow up period. One of the six AQs measured was associated with increased IV antibiotic usage in the follow up period. A higher number of IV antibiotic days for pulmonary exacerbations were observed in the presence of detectable levels of sputum C9-PQS. However, plasma levels of C9-PQS levels were not significantly associated with IV antibiotic usage. This may be explained by the low number of participants with detectable C9-PQS in plasma (eight adults compared to forty eight adults with detectable C9-PQS in sputum).

P. aeruginosa is the major respiratory pathogen in people with CF and is difficult to eradicate from the CF airways as it is intrinsically resistant to many classes of antibiotics and forms antibiotic resistant biofilms [303]. The AQ class of quorum sensing molecules plays an important role in pathogenicity for *P. aeruginosa* and AQ deficient mutants show reduced virulence in infection models [266, 304, 305]. PQS and its immediate precursor HHQ are the major AQ signalling molecules in *P. aeruginosa* [306]. PQS regulates the expression of at least 182 genes including those that code for the iron-chelating siderophores, pyoverdine and pyochelin [245, 281] as well as playing a role in regulating antibiotic resistance and biofilm maturation [246, 263, 283]. In addition, PQS regulates the production of key virulence factors that are associated with pulmonary exacerbations such as elastase [307], pyocyanin [308] and cyanide [309]. Both C9-PQS and NHQ are as effective as their C7 congeners PQS and HHQ at activating the AQ receptor PqsR which further drives the autoinduction of AQ biosynthesis and up regulates key virulence determinants [310]. Furthermore, molecular imaging techniques have shown that initial biofilm formation is marked by a dramatic increase in the production of C9-PQS, suggesting it may be important for the growth of *P. aeruginosa* in communities and early biofilm formation [311]. Although our

current understanding of the role of C9-PQS is limited [312], it is biologically plausible that C9-PQS may be associated with increased antibiotic usage due to increased virulence factor production during pulmonary exacerbations.

Recurrent severe pulmonary exacerbations are associated with both increased morbidity and mortality in CF [313]. In recent years, attempts to develop new classes of antimicrobial agents have included targeting of virulence factors or virulence regulatory mechanisms. Consequently, the AQ signalling system is a promising potential target for antimicrobial agents which do not kill the organism but instead block or attenuate the ability to cause disease. This is important as multiple courses of antibiotics are detrimental to the host and contribute to a growing global burden of multi-antibiotic resistance that needs to be addressed urgently.

2.4.1 Limitations

There are a number of limitations in this study that should be considered when interpreting these data. This is a retrospective analysis and the number of participants who died or had lung transplantation resulted in small numbers. We primarily assessed AQ levels as dichotomous; detectable or not detectable, as the variability across the sample size would have provided low power. Intra-subject variability of AQ concentrations is unknown and a single measure of AQ concentration may not reflect the longer period during which the AQs may influence disease progression. Whilst effect sizes are robust, findings are to be regarded as 'hypothesis generating' as significant *p* values may be a consequence of multiple hypothesis testing.

Despite limitations, these findings provide evidence of a possible association between both sputum and plasma C9-PQS levels and antibiotic usage, which should be confirmed through prospective study design.

2.4.2 Summary

In conclusion, this hypothesis generating prospective cohort study showed an association between C9-PQS detected in the sputum and increased antibiotic usage in

the CF population, which requires more comprehensive investigation to confirm or refute these findings. However, there were no other associations between the five AQs detected and adverse clinical outcomes measured. There is much to learn about AQ regulation in the clinical setting, particularly as development of anti-virulence drugs that target PQS-dependent QS pathways progresses [221].

2.4.3 Author Contributions

I would like to acknowledge the below co-authors that contributed towards data interpretation, data presentation and writing of the manuscript which was published in the Journal of Medical Microbiology, October 2018.

Dr Andrew Fogarty; Professor David A. Barrett; Dr Edward F. Nash; Dr Joanna L Whitehouse; Professor Alan R. Smyth; Dr Iain Stewart; Professor Alan Knox, Professor Paul Williams; Mr Nigel Halliday; Professor Miguel Cámara and Dr Helen Barr.

Chapter 3. Exploring the role of 2-Alkyl-4-quinolone quorum sensing molecules as biomarkers for culture-independent Pseudomonas aeruginosa burden in adults with cystic fibrosis

3.1 Introduction

This chapter investigates the correlation between 2-alkyl-4 quinolones (AQ) levels and live *P. aeruginosa* load in adults with CF using a viable cell separation technique followed by quantitative *P. aeruginosa* polymerase chain reaction (qPCR).

3.1.1 Background

P. aeruginosa is a gram-negative bacterium extremely well adapted to the airway niche in people with cystic fibrosis (CF). *P. aeruginosa* is a dominant pathogen in the CF lung and is associated with increased morbidity and mortality in this population [157]. Treatment and eradication of *P. aeruginosa* is a challenge as it is intrinsically resistant to many classes of antibiotics, produces a host of virulence factors and forms impenetrable biofilms in the CF airways [298].

P. aeruginosa controls the production of virulence factors using a cell-to-cell communication mechanisms known as quorum sensing (QS) [299]. One of the *P. aeruginosa* QS system is based on the production and sensing of 2-alkyl-4 quinolones (AQs), which control virulence in a population dependent manner [235, 239, 277, 314]. These AQs are only produced by *P. aeruginosa* and several closely related species [315].

Our previous studies have shown that several AQs are detectable in the sputum, plasma and urine of adults with CF and chronic pulmonary *P. aeruginosa* [280, 300].

Concentrations of two AQs, HHQ (2-heptyl-4-hydroxyquinoline) and HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) were most strongly associated with *P. aeruginosa* load measured by routine sputum culture at clinical stability [280]. Following intravenous anti-pseudomonal antibiotics for a pulmonary exacerbation, the concentrations of NHQ (2-nonyl-4-hydroxyquinoline) and HHQ in the plasma declined significantly, but there was no change in *P. aeruginosa* load, measured by routine culture [300].

In this study, we compared *P. aeruginosa* load measured historically by culture with live *P. aeruginosa* load quantified by polymerase chain reaction (qPCR). By using a photoreactive dye, propidium monoazide (PMA), we were able to differentiate between viable and compromised *P. aeruginosa* cells. With this modification, only viable (live *P. aeruginosa*) cells are amplified and quantified during the qPCR process [316].

3.1.2 Aims

We aimed to explore whether three previously analysed AQs; HHQ NHQ, and HQNO, correlated more strongly with new culture-independent measures of live *P. aeruginosa* load compared to a historical culture-dependent method. We tested correlations of these three AQs previously detected in sputum, plasma, and urine with *P. aeruginosa* in the sputum at stability and in response to treatment for a pulmonary exacerbation.

3.2 Methods

3.2.1 Participants and study design

In 2009-2011, spontaneous sputum samples were obtained and stored from adults with CF who had participated in two AQ biomarker studies, details of which have been previously published (9, 10).. In summary, spontaneous sputum, plasma and urine samples were obtained from 75 adults with CF at clinical stability and from 48 adults

at the start and end of intravenous (IV) antibiotic treatment for pulmonary exacerbation, according to Rosenfeld criteria [317]. Adults aged 16 to 60 were recruited from two UK adult CF centres from 2009-2011, who were known to have previously isolated *P. aeruginosa* from respiratory samples obtained during routine clinical practice. Spontaneous sputum, 8 mL venous blood and 25 mL urine samples were obtained at stability or within 72 h of the start and end of IV antibiotic therapy. In the original studies, baseline demographic data were collected and matched duplicate sputum plugs were frozen at -80°C for future studies

3.2.2 Quantitative microbiological analysis

In the previous biomarker studies, sputum plugs were mixed with an equal volume of dithiothreitol and diluted with 0.9% w/v saline and 100 µL of $\times 10^{-2}$ and $\times 10^{-4}$ solutions were plated on Pseudomonas isolation agar (Cetrimide Agar, Difco; BD, Sparks, MD, USA) which is used for the selective isolation and identification of *Pseudomonas aeruginosa*. The plates were incubated at 37 °C and colony counts were performed daily between 24 and 72 h, until maximal growth was achieved.

3.2.3 PMA-based qPCR analysis

For this study, the stored duplicate sputum plugs were analysed with PMA followed by quantitative PCR. The PMA molecules to penetrate only dead/compromised bacterial cells as they were impermeable to intact cell membrane. The PMA molecules were then fixed to DNA by 15 min exposure to LED blue light (IB-Applied Science, Spain). Upon exposure to PMA and light, permanent DNA modification was achieved by formation of stable covalent nitrogen-carbon bond, which prevented amplification (of DNA from dead/compromised cells) during qPCR analysis. Cells were pelleted at 10000 x g for 5 min prior to DNA extraction.

DNA extraction was conducted using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich Co. Ltd., Dorset, UK) with the following modifications. Samples were initially mixed with lysozyme (200 µL; 45 mg/mL, Sigma-Aldrich Co. Ltd., Dorset, UK) suspended in Gram-Positive Lysis Solution (included in the kit) followed by insertion

of glass beads. Cell disruption was then achieved by agitation in a Fastprep-24 Instrument (MP Biomedicals Europe, Illkirch, France) at 6.5 m/s for 60 sec twice and subsequently incubated at 37°C for 30 min. Further steps remained unchanged, and the DNA was resuspended in 50 µL of Elution Solution (included in the kit). DNA concentrations were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK). *P. aeruginosa*-specific qPCR assay was performed as described previously [318]. The lower limit of quantification (LLOQ) for *P. aeruginosa* in this assay was 100 CFU/g of sputum aliquot. Quantitative values were generated by Rotor Gene Q-series software (Qiagen, Crawley, UK) and expressed in CFU/g of sputum aliquot.

3.2.4 Sample processing for quantitative culture and AQ analyses

All sample processing for quantitative culture, differential cell counts and AQ analyses was performed in the initial AQ biomarker studies as previously described (9, 10). In summary, prepared clinical samples for AQ analyses were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Ortori *et al* [301]. Further information regarding the methodology of AQ analyses and the calculated lower limits of quantification (LLOQ) are provided in Chapter 2. To reduce multiple hypotheses testing, only the three AQs which were most strongly associated with *P. aeruginosa* burden from the previous study were analysed [280, 300]: HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline) and HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide).

3.2.5 Statistical analysis

Any value that was under the LLOQ was designated as undetected. AQ levels and P. aeruginosa qPCR loads were analysed after the addition of 1 to each value followed by log10 transformation to scale the data for interpretation. For analysis of absolute difference between two time points (at the beginning and end of IV antibiotics for pulmonary exacerbation), no log transformation was performed on the values. When

paired t-tests were used, normality of the data distribution was assessed both visually and using Shapiro-Wilk Test of Normality.

Comparisons of FEV1 measurements, sputum neutrophil concentrations, *P. aeruginosa* load measured by qPCR and *P. aeruginosa* load measured by culture at the beginning and end of IV antibiotics were analysed using paired t-tests. Quantitative loads of P. aeruginosa from culture and qPCR were assessed using Spearman's rank correlations, as well as sputum *P. aeruginosa* qPCR data with HHQ, NHQ and HQNO measured in blood, sputum and urine. Correlation coefficients of *P. aeruginosa* measured by culture and qPCR were transformed from r values to z scores using Fisher Z transformation to identify whether the z test statistic was outside the critical value, defined using two-tailed alpha 0.05 (16). Post-IV antibiotics changes in absolute FEV1 measurements, *P. aeruginosa* load (qPCR and culture) and AQ levels were calculated using the values at the end of IV antibiotics minus the values at the beginning of IV antibiotics. All statistical analyses were performed using Stata SE15 statistical software (Texas, USA).

3.3 Results

Sputum aliquots from 75 adults at clinical stability and 48 adults at the start and end of IV antibiotic treatment were available and subsequently thawed and processed for qPCR analysis. These samples did not detect *Burkholderia* sp. or *Acinetobacter* sp. based on the 16S rRNA gene sequencing data we obtained. Baseline demographics of participants included in the analysis at clinical stability and exacerbation are summarised in Table 3-1.

Table 3-1. Baseline demographics of participants at clinical stability (n=75) and exacerbation (n=48).

Variable	Baseline
Clinical stability cohort	n=75
Age in years: median (range)	38.6 (17.8 to 61.5)
Gender, males (%)	49 (65.3)
FEV_1 % predicted: mean (SD)	55 (±18)
Absolute FEV_1 in L: mean (SD)	2.0 (±0.8)
BMI: mean (SD)	22.9 (± 3.3)
P. aeruginosa status by culture at baseline: n (%)	
Never	0 (0)
Free	1 (1.3)
Intermittent	2 (2.7)
Chronic	72 (96)
Exacerbation cohort	n=48
Age in years: median (range)	27.5 (17 to 59)
Gender, males (%)	27 (56.3)
FEV_1 % predicted: mean (SD)*	46.6 (±16)
Absolute FEV_1 in L: mean (SD)*	1.7 (±0.7)
BMI: mean (SD)	21.9 (± 3.9)
Diagnostic microbiology results: n (%)	
P. aeruginosa isolated only:	33 (68.8)
P. aeruginosa co-infection with:	11 (22.9)
MSSA	4 (36.4)
MRSA	1 (9.1)
Haemophilus influenza and *MSSA*	1 (9.1)
Aspergillus fumigatus and *MSSA*	1 (9.1)
Candida albicans	4 (36.4)
No *P. aeruginosa* isolated:	4 (8.3)
MSSA	1 (25)
MRSA	2 (50)
Respiratory commensals	1 (25)

n = number of participants with data available; SD = standard deviation, *; spirometry at exacerbation. *P. aeruginosa* status of participants defined by Leeds criteria [169].

3.3.1 Individual AQ molecules are associated with live *P. aeruginosa* qPCR load at clinical stability compared to *P. aeruginosa* culture load

At clinical stability, there was no correlation between live *P. aeruginosa* load measured by qPCR and the load detected by culture (r=0.174, *p*=0.166, Figure 3-1). All of the three AQs (HHQ, NHQ, HQNO) consistently correlated with live *P. aeruginosa* load detected by qPCR in all sample types (sputum, plasma and urine, Figure 3-2).

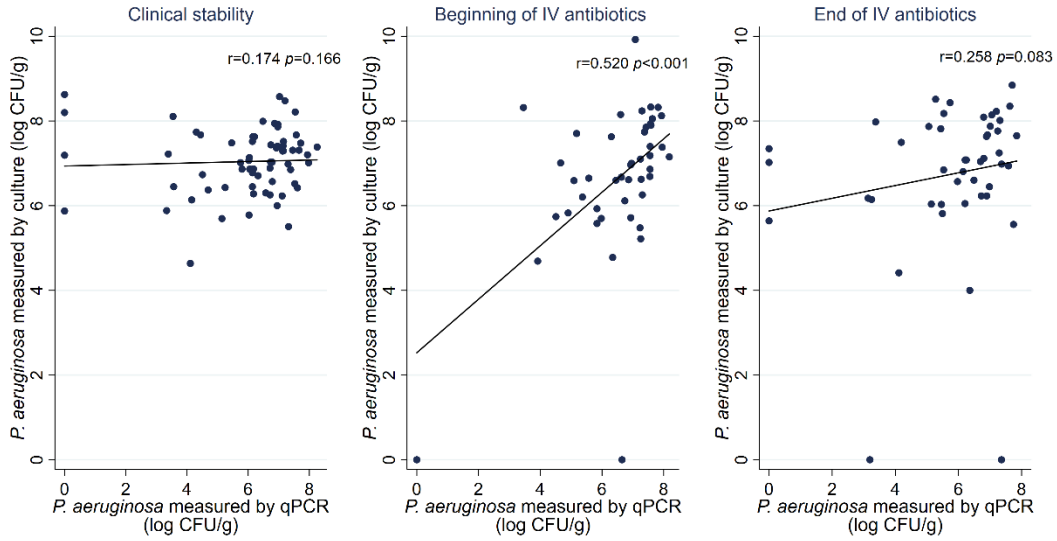

Figure 3-1. The correlation between *P. aeruginosa* loads in sputum quantified by qPCR and culture (at stability, exacerbation and post-exacerbation).
Figure 1 legend: Clinical stability n=65; Beginning of IV antibiotics for pulmonary exacerbation n=45; End of IV antibiotics for pulmonary exacerbation, n=46, where n is the number of patients with samples available for analysis. r; Spearman's Correlation Coefficient.

The strongest relationship with live *P. aeruginosa* qPCR load was observed with plasma HHQ (r=0.550, r^2= 0.30, *p*<0.001, Figure 3-2).

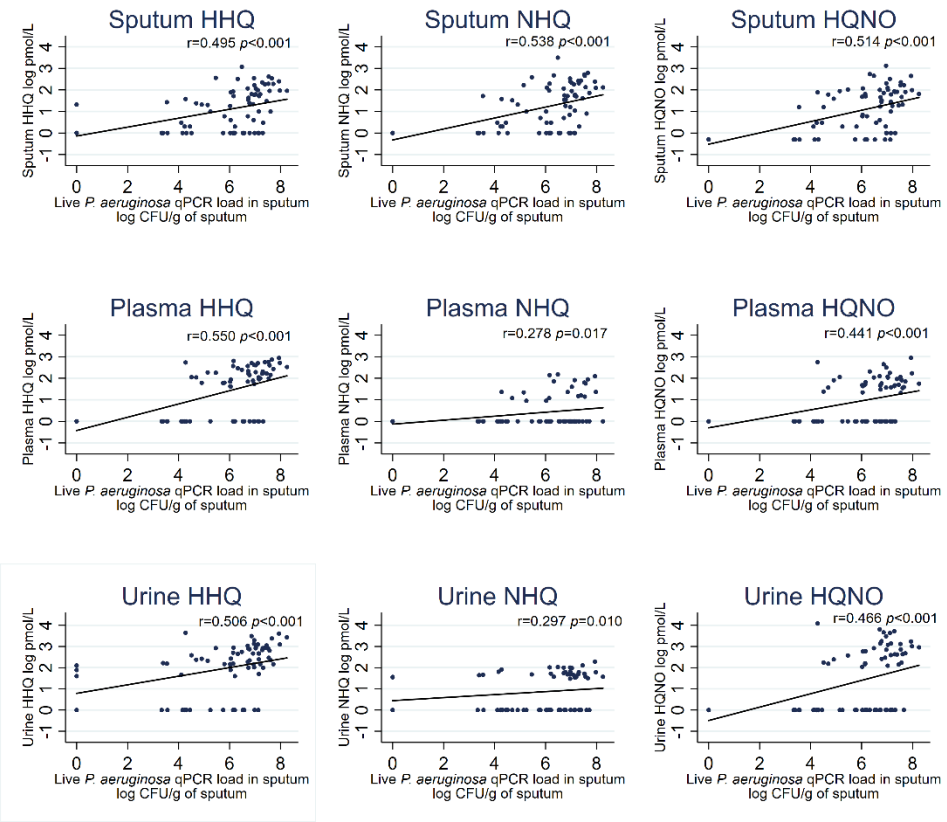

Figure 3-2. Correlations of HHQ, NHQ and HQNO detected in sputum, plasma and urine with live *P. aeruginosa* load by qPCR in sputum during clinical stability.
Figure 3-2 legend: Median (interquartile range) for sputum live *P. aeruginosa* load by qPCR was 3.3 x 10^6 CFU/g (1.4 x 10^5 – 1.4 x 10^7 CFU/g) of sputum. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline. HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide; Sputum, n=75; plasma, n=74; urine, n=75, where n is the number of participants with data available for statistical analysis. Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol/L), NHQ (10, 10 pmol/L) and HQNO (30, 30 pmol/L).No LLOQ was defined for sputum AQs, The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells/g of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation.

In contrast, *P. aeruginosa* quantitative load measured by culture only correlated with HHQ and NHQ measured in sputum (r=0.381, *p*=0.002; r=0.296, *p*=0.017) and urine (r= 0.264, *p*=0.034; r=0.322, *p*=0.012), respectively (Table 3-2). No correlations were observed with any AQs measured in plasma.

Comparison of the correlation coefficients of *P. aeruginosa* load measured by qPCR against culture demonstrated significantly higher correlations on qPCR compared to

culture in sputum NHQ and HQNO, plasma HHQ and HQNO, and urine HHQ and HQNO (Table 3-2).

Table 3-2. Correlations of HHQ, NHQ and HQNO in sputum, plasma and urine with live *P. aeruginosa* loads measured by qPCR at clinical stability compared to historical quantitative *P. aeruginosa* load by culture.

		Sputum	Plasma	Urine
	AQ	Spearman's Correlation Coefficient for qPCR, r (p-value)+		
qPCR	**HHQ**	0.495 (<0.001)*	0.550 (<0.001)*	0.506 (<0.001)*
	NHQ	0.538 (<0.001)*	0.278 (0.017)*	0.297 (0.010)*
	HQNO	0.514 (<0.001)*	0.441 (<0.001)*	0.466 (<0.001)*
		Spearman's Correlation Coefficient for Culture, r (p-value)β		
Culture	**HHQ**	0.381 (0.002)*	0.232 (0.065)	0.264 (0.034)*
	NHQ	0.296 (0.017)*	0.121 (0.342)	0.311 (0.012)*
	HQNO	0.203 (0.104)	0.169 (0.183)	0.197 (0.115)
		Fisher Z transformation qPCR vs Culture (p-value) ¥		
qPCR vs culture	**HHQ**	0.818 (0.207)	2.187 (0.014)*	1.661 (0.048)*
	NHQ	1.708 (0.044)*	0.940 (0.174)	-0.088 (0.465)
	HQNO	2.090 (0.018)*	1.733 (0.042)*	1.760 (0.039)*

Median (interquartile range) for sputum live *P. aeruginosa* load by qPCR was 3.3 x 10^6 CFU/g (1.4 x 10^5 – 1.4 x 10^7 CFU/g) of sputum. Median (interquartile range) for sputum *P. aeruginosa* load by culture was 1.2 x 10^7 CFU/g (2.8 x 10^6 – 3.28 x 10^7 CFU/g) of sputum. AQ: 2-Alkyl-4-quinolone, HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline; HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide. Analysis with qPCR: Sputum, n=75; plasma, n=74; urine, n=75, Analysis with Culture: Sputum, n=65; plasma, n=64; urine, n=65 where n is the number of patients with samples available for analysis. Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol/L), NHQ (10, 10 pmol/L) and HQNO (30, 30 pmol/L). No LLOQ was defined for sputum AQs, The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells/g of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation. ¥;Z test comparing Spearman's Coefficient correlation values in qPCR and culture. *significant at $p<0.05$

3.3.2 Clinical and microbiological changes in response to treatment for a pulmonary exacerbation

There was a significant correlation between live *P. aeruginosa* load measured by qPCR and the load detected by culture at the beginning of IV antibiotics for pulmonary

exacerbation (r=0.520, *p*=<0.001). Correlation was not observed when IV antibiotics were stopped (r=0.258, *p*=0.083, Figure 3-1).

Following treatment for a pulmonary exacerbation, the mean absolute FEV_1 increased from 1.68 L (SD ±0.68) at the beginning of IV antibiotics to 1.96 L (SD ± 0.82) at the end of IV antibiotics, (*p*=<0.0001). Mean percent (%) predicted FEV_1 increased from 46.6% (SD ± 16) to 52.4% (SD ± 17.8), (*p*=<0.0001, Table 3-3).

The mean number of neutrophils detected in sputum significantly reduced following IV antibiotic treatment for a pulmonary exacerbation, from 7.15 log cells/g (SD ± 0.37) to 6.9 log cells/g (SD ± 0.38), (*p*<0.001). Mean *P. aeruginosa* load detected by culture showed no significant change between the beginning of IV antibiotics; 6.59 log_{10}CFU/g (SD ± 1.83) and the end of IV antibiotics; 6.67 log_{10}CFU/g (SD ± 1.82), (*p*=0.84). Mean live *P. aeruginosa* load measured by qPCR showed a significant reduction from 6.40 log_{10}CFU/g (SD ± 1.52) at the beginning of IV antibiotics, to 5.71 log_{10}CFU/g (SD ± 1.97) at the end of IV antibiotics (*p*=<0.001, Table 3-3).

Table 3-3. Comparison of Clinical and microbiological factors between the beginning and end of IV antibiotics for pulmonary exacerbation.

Variable	Beginning of IV antibiotics: mean (SD)	End of IV antibiotics : mean (SD)	p-value§
Absolute FEV1/L	1.68 L (±0.68)	1.96 L (±0.82)	<0.0001
FEV1 % predicted	46.6% (±16)	52.4% (±17.8),	<0.0001
Sputum neutrophils*	7.15 $\log_e$cells/g (±0.37)	6.9 $\log_e$cells/g (±0.38)	<0.001
P. aeruginosa load culture¥	6.59 $\log_{10}$CFU/g (±1.83)	6.67 $\log_{10}$CFU/g (±1.82)	0.84
P. aeruginosa load PCR¥	6.40 $\log_{10}$CFU/g (±1.52)	to 5.71 $\log_{10}$CFU/g (±1.97)	<0.001

*; log cells/g, ¥; log10 CFU/g, §; paired t test.

Changes in absolute FEV_1 measurements and live *P. aeruginosa* measured by qPCR were negatively correlated (r= -0.415, *p*= 0.004, Figure 3-3*).* There was no correlation between changes in absolute FEV_1 and *P. aeruginosa* measured by culture (r=0.115, *p*=0.473, Figure 3-3).

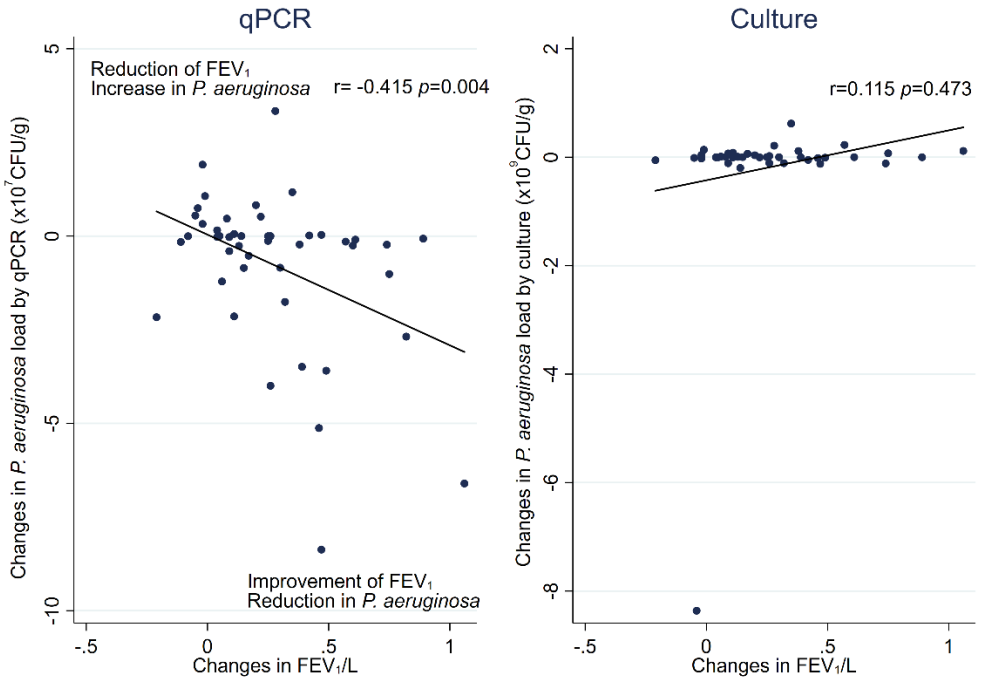

Figure 3-3. **The correlation between changes of FEV_1/L and changes of sputum *P. aeruginosa* load measured by qPCR and culture following treatment for a pulmonary exacerbation.**
Figure 3 legend: Differences between absolute FEV_1 and *P. aeruginosa* load were calculated using the values at the end of IV antibiotics for pulmonary exacerbation minus the values at the beginning of IV antibiotics. A positive FEV_1 change reflects improvement of FEV_1 and a negative *P. aeruginosa* change reflects a reduction in *P. aeruginosa* load after IV antibiotic therapy. *p*-value derived from Spearman's rank correlations.

3.3.3 HHQ and NHQ concentrations are associated with live *P. aeruginosa* PCR load before and after pulmonary exacerbation

At the start of pulmonary exacerbation HHQ, NHQ and HQNO concentrations measured in sputum, plasma and urine showed positive correlations with live *P. aeruginosa* load measured by qPCR (*p*=<0.001, Table 3-4). The strongest correlation was with sputum NHQ (r=0.736, r^2=0.54, *p*<0.001). *P. aeruginosa* load measured by qPCR are consistent with *P. aeruginosa* load measured previously by culture with the exception of urinary HQNO. Correlations between HHQ and NHQ measured in the urine were significantly stronger with *P. aeruginosa* qPCR when compared to culture (Table 3-4).

At the end of antibiotic treatment, HHQ levels were positively correlated with live *P. aeruginosa* load measured by qPCR in all sample types (sputum r=0.527, $p<0.001$; plasma r=0.430, p<0.001; urine r=0.509, $p<0.001$, Table 3-4). Significant correlations of live *P. aeruginosa* load measured by qPCR were observed in sputum NHQ (r=0.554, $p=0.001$), sputum HQNO (r=0.583 $p<0.001$) and urine HQNO (r=0.411 $p=0.004$) but not in plasma (Table 3-4). These findings are consistent with previous *P. aeruginosa* loads measured in culture with the exception of plasma HHQ and urine HQNO which showed significant correlations with qPCR load but not culture.

Table 3-4. Correlations of HHQ, NHQ and HQNO concentrations in sputum, plasma and urine with sputum live *P. aeruginosa* load measured using qPCR at the start and end of IV-antibiotic treatment for a pulmonary exacerbation compared to historical culture data.

	Start of IV antibiotics§			End of IV antibiotics§		
	qPCR	Culture	qPCR vs culture	qPCR	Culture	qPCR vs culture
AQ	Spearman's Correlation Coefficient, r (*p*-value)		Z (*p*-value)¥	Spearman's Correlation Coefficient, r (*p*-value)		Z (*p*-value)¥
HHQ						
-Sputum	0.626 (<0.001)*	0.522 (<0.001)*	0.725 (0.234)	0.527 (<0.001)*	0.345 (0.019)*	1.059 (0.145)
-Plasma	0.524 (<0.001)*	0.546 (<0.001)*	-0.141 (0.444)	0.430 (0.003)*	0.121 (0.430)	1.570 (0.058)
-Urine	0.607 (<0.001)*	0.306 (0.041)*	1.810 (0.035)*	0.509 (<0.001)*	0.295 (0.047)*	1.207 (0.114)
NHQ						
-Sputum	0.736 (<0.001)*	0.532 (<0.001)*	1.627 (0.052)	0.554 (<0.001)*	0.297 (0.045)*	1.486 (0.069)
-Plasma	0.452 (0.001)*	0.314 (0.038)*	0.746 (0.228)	0.234 (0.113)	0.103 (0.500)	0.625 (0.228)
-Urine	0.636 (<0.001)*	0.350 (0.019)*	1.799 (0.036)*	0.248 (0.090)	0.216 (0.150)	0.157 (0.438)
HQNO						
-Sputum	0.600 (<0.001)*	0.431 (0.003)*	1.082 (0.140)	0.583 (<0.001)*	0.294 (0.047)*	1.707 (0.044)*
-Plasma	0.577 (<0.001)*	0.479 (0.001)*	0.630 (0.264)	0.206 (0.166)	0.115 (0.453)	0.432 (0.333)
-Urine	0.524 (<0.001)*	0.273 (0.070)	1.404 (0.273)	0.411 (0.004)*	0.222 (0.138)	0.988 (0.162)

§; Median (interquartile range) for sputum live *P. aeruginosa* load was 8 x 10^6 CFU/g (3 x 10^3 – 2 x 10^8 CFU/g) at pre-antibiotics and 2 x 10^6 CFU/g (1 x 10^3 – 7 x 10^7 CFU/g) at post-antibiotics. ¥; AQ: 2-Alkyl-4-quinolone, HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline.HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide. qPCR: Sputum, n=48; plasma, n=47; urine, n=48. Culture: Sputum, n=45; plasma, n=44; urine, n=45, (where n is the number of patients with samples available for analysis). Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol/L), NHQ (10, 10 pmol/L) and HQNO (30, 30 pmol/L). No LLOQ was defined for sputum AQs, The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells/g of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation. ¥; Z test comparing Spearman's Coefficient correlation values in qPCR and culture. *significant comparison at p<0.05

3.3.4 Changes in live *P. aeruginosa* load measured using qPCR positively correlated with changes in NHQ signal concentration in plasma

Further analyses were performed to investigate the relationship between absolute changes of live *P. aeruginosa* measured by qPCR with changes in HHQ, NHQ and HQNO concentrations following IV antibiotics for a pulmonary exacerbation. A positive correlation was observed between change in plasma NHQ following systemic antibiotics and changes in live *P. aeruginosa* loads measured by qPCR in sputum (r=0.422, *p*=0.003, Table 3-5, Figure 3-4). No other significant correlations were found between differences in absolute live *P. aeruginosa* qPCR load and changes in NHQ, HHQ or HQNO concentrations (NHQ in sputum and urine; HHQ and HQNO in sputum, plasma and urine). There were no correlations with changes in *P. aeruginosa* measured by culture and changes with any AQ in all sample types (Table 3-5).

Table 3-5. Correlations between changes in sputum, plasma and urine AQ concentrations with live *P. aeruginosa* load changes after IV antibiotic treatment.

Changes post-IV antibiotics		qPCR	Culture	qPCR vs culture
AQ		Spearman's Correlation Coefficient, r (*p*-value)		Z (*p*-value)¥
HHQ				
-	Sputum	0.252 (0.083)	0.266 (0.085)	-0.065 (0.474)
-	Plasma	0.276 (0.061)	0.285 (0.067)	-0.047 (0.481)
-	Urine	0.182 (0.215)	-0.042 (0.787)	1.044 (0.148)
NHQ				
-	Sputum	0.237 (0.104)	0.202 (0.193)	0.169 (0.433)
-	Plasma	0.422 (0.003)*	0.230 (0.143)	0.917 (0.180)
-	Urine	0.274 (0.059)	0.082 (0.602)	0.982 (0.163)
HQNO				
-	Sputum	0.230 (0.115)	0.148 (0.345)	-0.001 (0.499)
-	Plasma	0.125 (0.404)	0.125 (0.431)	0.395 (0.346)
-	Urine	0.205 (0.163)	-0.109 (0.488)	1.457 (0.073)

Median (interquartile range) for sputum live *P. aeruginosa* qPCR load was 8 x 10^6 CFU/g (5 x 10^5 – 3 x 10^7 CFU/g) at pre-antibiotics and 2 x 10^6 CFU/g (2 x 10^5 – 1 x 10^7 CFU/g) post-antibiotics. Median (interquartile range) for culture *P. aeruginosa* load was 7 x 10^6 CFU/g (8 x 10^5 – 6 x 10^7 CFU/g) at pre-antibiotics and 1 x 10^7 CFU/g (2 x 10^6 – 7 x 10^7 CFU/g) post-antibiotics. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline. HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide. qPCR: Sputum, n=48; plasma, n=47; urine, n=48, Culture: Sputum, n=43; plasma, n=42; urine, n=48, (where n is the number of patients with samples available for analysis) Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol/L), NHQ (10, 10 pmol/L) and HQNO (30, 30 pmol/L). No LLOQ was defined for sputum AQs, The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells/g of sputum. Values under LLOQ were designated a 0. ¥; Z test comparing Spearman's Coefficient correlation values in qPCR and culture. *; p-value<0.05

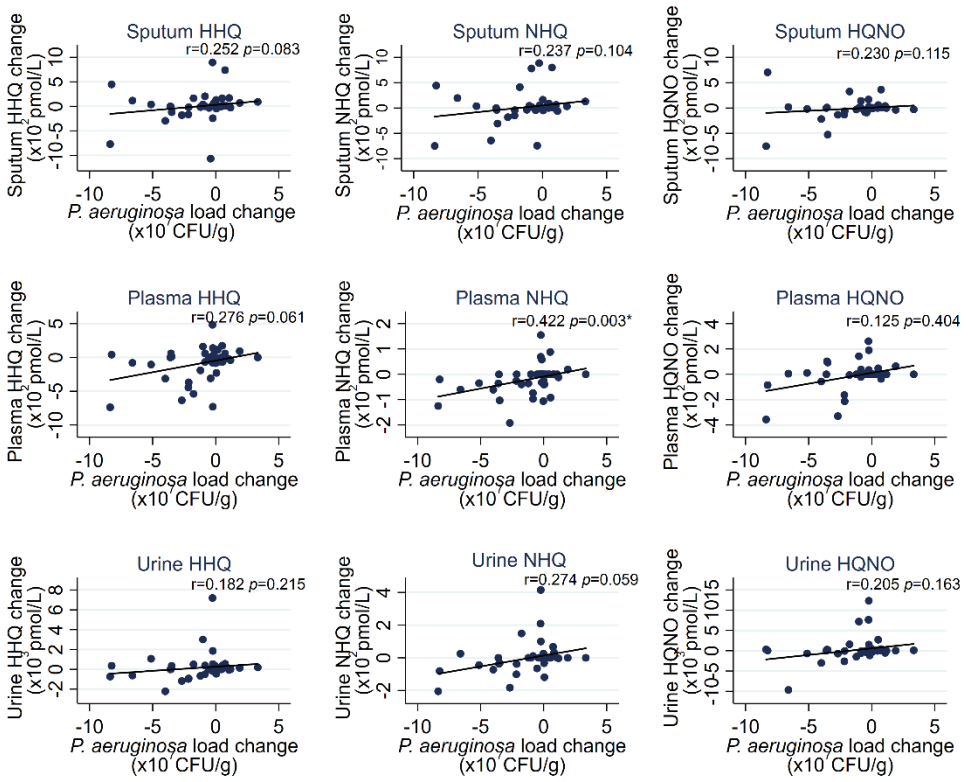

Figure 3-4. Individual changes of HHQ, NHQ and HQNO concentrations in sputum, plasma and urine with live *P. aeruginosa* load changes between the beginning and end of IV antibiotics.

Figure 3-4 legend: Median (interquartile range) for sputum live *P. aeruginosa* qPCR load was 8 x 10^6 CFU/g (5 x 10^5 – 3 x 10^7 CFU/g) at pre-antibiotics and 2 x 10^6 CFU/g (2 x 10^5 – 1 x 10^7 CFU/g) post-antibiotics. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline. HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide. Sputum, n=48; plasma, n=47; urine, n=48 (where n is the number of patients with samples available for analysis Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol/L), NHQ (10, 10 pmol/L) and HQNO (30, 30 pmol/L). No LLOQ was defined for sputum AQs, The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells/g of sputum. Values under LLOQ were designated a 0.

3.4 Discussion

This is the first study to explore systemic measurements of AQ quorum sensing molecules as biomarkers of live *P. aeruginosa* load measured by qPCR in the sputum in adults with CF. Overall, there were stronger correlations between AQ concentrations and qPCR measures of bacterial load compared to previous culture methodology which is likely to be less standardised therefore contributing to higher levels of measurement error. In contrast to *P. aeruginosa* load measured by culture at clinical stability, qPCR *P. aeruginosa* load showed consistent correlations with HHQ, NHQ and HQNO measured in sputum, plasma and urine. At the beginning of IV antibiotics for pulmonary exacerbation, HHQ and NHQ in sputum, plasma and urine were positively correlated with *P. aeruginosa* load measured by both qPCR and culture. In addition, absolute change of sputum *P. aeruginosa* load measured by qPCR were reflected by an absolute change in plasma NHQ between the start and end of IV antibiotics for pulmonary exacerbation.

We found no consistent correlations between culture and qPCR measures of *P. aeruginosa* load during stability or following IV antibiotic treatment, although correlations were observed at the beginning of IV antibiotics for a pulmonary exacerbation. The *P. aeruginosa* load measured by qPCR reduced significantly after antibiotic treatment whilst there was no significant difference using culture. It is possible that qPCR assays may be more sensitive at detecting *P. aeruginosa* compared to culture [319]. PMA-qPCR may more accurately quantify viable uncultivable bacterial cells which are metabolically dormant and therefore not detected by culture, as suggested by Deschaght *et al* [320] demonstrating the potential strengths of PMA-qPCR compared to culture. This theory supports our observations that the correlation between qPCR quantification of *P. aeruginosa* load and AQs were stronger compared to culture. However, we found higher levels of *P. aeruginosa* detected by culture compared to qPCR quantification. It is possible that the freeze thaw cycle may have reduced the viable *P. aeruginosa* in the sputum

samples that underwent qPCR analysis. Alternatively, quantification by culture may be subject to individual operator error and inaccuracy [321].

In our study, only *P. aeruginosa* load measured by qPCR was correlated with improvements in absolute FEV_1 following treatment for a pulmonary exacerbation, and not culture. There are conflicting data in the literature regarding the relationship between culture and culture-independent quantitative *P. aeruginosa* load measurements with CF related outcomes [174, 320, 322, 323]. For example, McLaughlin *et al* showed no correlation between lung function improvement and a reduction of *P. aeruginosa* load measured by culture after pulmonary exacerbation [323]. However, other studies have shown a significant reduction in culture-dependent and culture-independent measures of *P. aeruginosa* load after treatment for pulmonary exacerbation, and these correlated significantly with lung function improvement [320, 324]. These variable observations are likely to be a result of different methodological approaches in measuring the quantitative load of *P. aeruginosa*. Here, we further demonstrate the dynamic relationship of AQs with longitudinal changes in quantitative *P. aeruginosa* load, which may offer novel opportunities to monitor bacterial burden during clinical stability and acute exacerbation in CF.

3.4.1 Strengths

Strengths of this study include the large multicentre participant cohort and the pre-treatment of sputum with PMA which enabled only viable *P. aeruginosa* bacterial cells to be amplified and quantified. The sputum samples were collected and processed in line with conventional practice following a rigorous protocol, thereby reducing any variance in the signals generated by random error. In addition, the samples were homogenised and divided into aliquots before storage to avoid multiple freeze-thawing and loss of sample integrity. Furthermore, participants known to have previously isolated *Burkholderia cepacia complex* were excluded from the study as these organisms can also produce AQs [280, 300]. To limit multiple comparisons of different AQs, we assessed the three that were found to have the most promising

biomarker potential from previous studies: HHQ, NHQ, HQNO [280, 300], however the type 1 error rate was not controlled.

3.4.2 Limitations

There are a number of limitations in this study that should be considered when interpreting these data. The numbers of participants in this study were small and therefore the study may have been inadequately powered to show a true effect. Duplicate aliquots of sputum were frozen and thawed to perform *P. aeruginosa* qPCR analysis after the time-point of the quantification by culture. Therefore, this exposure to a longer period of frozen storage may limit direct comparisons between the burden of *P. aeruginosa* as measured by qPCR analysis and that quantified by culture colony forming units. This may account for the lack of correlation between the two different measures of *P. aeruginosa* load at clinical stability.

P. aeruginosa bacterial load measured using qPCR accounted for up to 30% (r^2=0.30) of the biomarker variability in AQs at clinical stability and 54% (r^2=0.54) at pulmonary exacerbation. Variability in AQ concentrations that was not completely attributable to *P. aeruginosa* load may limit its potential clinical use as a marker of specific bacterial burden in clinical practice. This wide range of variability may be attributable to additional factors not measured in this study that could influence AQ concentration including oxygen gradients and nutrient availability in the lung environment resulting in heterogeneous conditions, which may lead to variability in sputum AQ concentrations [325]. Furthermore, the distribution, half-life and clearance of AQs in different sample types *in vivo* is not known. For example, the collection of urine samples used a random “catch” method that may be associated with random error in the concentration of molecules being measured. To address the potential of urinary AQs as biomarkers, future studies should correct for differential dilution with measurement of urinary creatinine, which may enhance the sensitivity to detect effects that were promising but non-significant in the limited longitudinal sample. Whilst analyses do not adjust for demographic or clinical factors, we reduced the effect of between-person variability in paired tests. Lastly, this was a retrospective

study limited to adults with CF, further studies are needed to determine whether these findings are applicable to adults without CF and the wider CF population, including the paediatric CF population. Despite these limitations, there were associations between *P. aeruginosa* bacterial load in the sputum and systemic AQ concentrations in plasma and random urine samples, suggesting they may be useful in clinical practice as semi-quantitative, minimally-invasive biomarkers of infection.

3.4.3 Summary

In summary, further investigation is needed to determine the relationship between AQs and bacterial burden in newly acquired *P. aeruginosa* pulmonary infection, to determine its potential as a screening tool for early infection. A minimally-invasive diagnostic test for *P. aeruginosa* would be especially useful in young children who cannot always expectorate sputum and may avoid the need for more invasive tests such as bronchoalveolar lavage. In addition, surrogate systemic measures of *P. aeruginosa* burden may prove increasingly useful in the era of highly effective CTFR modulator therapy where spontaneous sputum production is likely to decrease in the longer term [326].

In conclusion, dynamic changes in the bacterial load of *P. aeruginosa* objectively measured by qPCR were sensitive to the clinical course of exacerbation and intervention, which were not apparent from culture methods. AQs concentrations were more strongly correlated with culture independent methods of *P. aeruginosa* compared to culture dependent techniques. HHQ, NHQ and HQNO measured in sputum, plasma and urine were positively correlated with *P. aeruginosa* load measured by qPCR in sputum at pulmonary exacerbation and at clinical stability. In addition, changes in plasma NHQ reflect a reduction in *P. aeruginosa* after IV antibiotic treatment for pulmonary exacerbation, which has not been previously demonstrated. Further prospective investigation should seek to understand the sources of clinical variability in systemic AQ quorum sensing molecules, and confirm

whether detection in plasma or urine can be reliably used as a minimally-invasive biomarker of *P. aeruginosa* load in people with CF.

3.4.4 Author contributions

I would like to acknowledge the below co-authors that contributed towards data interpretation, data presentation and writing of the manuscript which was accepted for publication in the Journal of Medical Microbiology, August 2021.

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Chapter 4. Novel detection of specific bacterial quorum sensing molecules in saliva: potential non-invasive biomarkers for pulmonary Pseudomonas aeruginosa in cystic fibrosis

4.1 Introduction

This chapter describes a study aimed to determine whether AQs could be detected in saliva of patients with CF and known infection with *P. aeruginosa*. Saliva and sputum samples were obtained from 89 adults with CF and analyzed using liquid chromatography-tandem mass spectrometry. This is an important potential area of research as saliva can be easily collected and stored by patients for research purposes, and also collected and posted to clinical centres where rapid analysis may guide timely treatments.

4.1.1 Background

P. aeruginosa is a dominant respiratory pathogen in the CF lung and is associated with increased morbidity and mortality in this population [327]. Regular microbiological surveillance of the CF airways is important for the early detection of *P. aeruginosa* to facilitate timely and targeted antimicrobial therapy [163]. However, it is difficult to diagnose pulmonary *P. aeruginosa* in people with CF who are unable to spontaneously produce sputum samples. More invasive procedures such as broncho-alveolar lavage or sputum induction are rarely suitable for clinical surveillance programmes. Current clinical practice uses cough swabs, but these lack sensitivity compared to sputum induction and cannot reliably exclude pulmonary *P. aeruginosa* [328]. Finding the best way to diagnose and treat *P. aeruginosa* has also

been identified as one of the top 10 research priorities by the James Lind Alliance priority setting partnership in CF [329].

P. aeruginosa uses cell-to-cell signalling systems known as quorum sensing (QS) to regulate the production of biofilms and virulence factors [299, 330]. The *pqs* QS system produced by *P. aeruginosa* uses multiple 2-alkyl-4 quinolones (AQs) as signal molecules. Six AQs are detectable in the sputum, plasma and urine of adults with CF and chronic pulmonary *P. aeruginosa* [279, 280]. Furthermore, concentrations of three of these AQs; HHQ 2-heptyl-4-hydroxyquinoline (HHQ), 2-nonyl-4-hydroxyquinoline (NHQ) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), are strongly associated with *P. aeruginosa* load measured by routine sputum culture at clinical stability [6].

4.1.2 Aims

The aims of this study were to determine whether:

- AQs are detectable in salivary samples obtained from adults with CF with known pulmonary *P. aeruginosa*
- To investigate if salivary AQ concentrations correlate with sputum quantitative load of *P. aeruginosa* measured by polymerase chain reaction (PCR).

4.2 Methods

4.2.1 Participants and Study design

Eighty-nine adults with CF were recruited at clinical stability to an AQ biomarker study, the full details of which were previously published [280]. In summary, adults aged 16 to 60 were recruited from two UK adult CF centres. All of the participants were known to have previously cultured *P. aeruginosa* from sputum samples and 85 (96%) were chronically infected as defined by the Leed's criteria [169]. Participants known to have previously isolated *Burkholderia cepacia complex* were excluded from the study, as these organisms can also produce AQs [280, 300].

4.2.2 Sample processing

Salivary samples were expelled into universal containers after mouth rinsing with hospital tap water. Spontaneous sputum samples were collected after the salivary samples were obtained according to standard guidelines [331]. Sputum plugs and saliva were stored in duplicate aliquots and frozen at -80°C, when sufficient volume was available to split the sample. Prepared clinical samples for AQ analyses were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Ortori *et al* [301].

A total of six AQs were analysed using LC-MS/MS based on their abundance in CF sputum: HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline), PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone), C9-PQS (2-nonyl-3-hydroxy-4(1*H*)-quinolone), HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) and NQNO (2-nonyl-4-hydroxyquinoline-*N*-oxide) [5, 6].

4.2.3 Polymerase chain reaction (PCR) analysis

Prior to DNA extraction, sputum samples were pre-treated with propidium monoazide (PMA) to bind dead/compromised bacterial cells [332]. With this modification, only viable (live *P. aeruginosa*) cells are amplified and quantified during the qPCR process

[316]. Further details on the AQ and qPCR methodology are described in Chapters 2 and 3.

4.2.4 Statistical Analysis

AQ concentrations and quantitative load of *P. aeruginosa* were analysed after the addition of 1 to each value followed by $\log_{10}$ transformation to scale the data for interpretation.

The concentrations of AQs in saliva, sputum and *P. aeruginosa* load were compared using Spearman rank correlation coefficients. Sensitivity, specificity, receiver operating characteristic (ROC), positive and negative predicted values of saliva AQs compared to *P. aeruginosa* in sputum measured by PCR were calculated. A McNemar's test was performed to ascertain the agreement of the two sampling techniques. Statistical significance was assessed as $p<0.05$. All data were analysed using Stata SE15 statistical software (Texas, USA).

4.3 Results

The majority of participants 85/89 (95.6%) were chronically colonised with *P. aeruginosa* [12]. Baseline demographics are summarised in Table 4-1. *P. aeruginosa* was detected in 70/75 (93.3%) sputum samples available for qPCR analysis.

Table 4-1. Baseline clinical characteristics and *P. aeruginosa* status of participants.

Variable	Baseline (n=89)
Nottingham University Hospitals NHS trust University Hospitals	46
Birmingham NHS Foundation Trust	43
Age in years: median (range)	28.3 (17.8 to 61.5)
Gender, males (%)	57 (64.0)
FEV_1 % predicted: mean (SD)	55.6 (±19.5)
Absolute FEV_1 in L: mean (SD)	2.1 (±0.9)
BMI: mean (SD)	22.9 (± 3.3)
P. aeruginosa status at baseline: n (%)	
Never	0 (0)
Free	1 (1.1)
Intermittent	3 (3.3)
Chronic	85 (95.5)

n = number of participants with data available; SD = standard deviation
P. aeruginosa status of participants defined by Leeds criteria [169]

Out of the 6 AQs investigated, at least one AQ was detected in 39/89 (43.8%) saliva samples and 70/77 (90.9%) sputum samples. Salivary AQs had a sensitivity of 50% (95%CI; 37.8;62.2), specificity of 100% (95%CI; 47.8;100), positive predictive value of 100% (95%CI; 90;100), negative predictive of value 12.5% (95%CI;4.2;26.8) compared to live *P. aeruginosa* load in sputum measured using PCR (Table 4-2).

Table 4-2. The sensitivity, specificity, positive predictive value and negative predictive value of saliva AQs with *P. aeruginosa* PCR measured in sputum (N=75).

	PsA PCR +	PsA PCR -	Total	
Saliva AQ +	35	0	35	PPV (95%CI); 100% (90.0;100.0)
Saliva AQ -	35	5	5	NPV (95%CI); 12.5%(4.2;26.8)
Total	70	5	75	McNemar's chi2; 35.00 (p<0.001)
	Sens (95%CI)	Spec (95%CI)		ROC area (95%CI)
	50 % (37.8;62.2)	100% (47.8;100.0)		0.75 (0.70;0.81)

AQ; 2-alkyl-4-quinolones, +; positive, -; negative, PsA; *Pseudomonas aeruginosa* PPV; positive predicted value, NPV; negative predictive value, Sens; sensitivity, Spec; specificity, ROC area; Receiving operator characteristic area curve (Sensitivity+Specificity/2)
Out of the original cohort of 89 participants, 75 sputum samples were available for qPCR analysis.

4.3.1 Correlation of AQs concentrations measured in saliva and spontaneous sputum

There were significant positive associations between all 6 AQs measured in spontaneous sputum and saliva samples (Table 4-3 and Figure 4-1).

Table 4-3. Correlations of AQs detected in saliva with AQs detected in spontaneous sputum and *P. aeruginosa* measured by PCR.

		Spearman's Correlations: r (*p*-value)			
AQ in saliva	N (%)	AQ in sputum*	95%CI	*P. aeruginosa* PCR°	95%CI
HHQ	34 (38)	0.65 (<0.001)	0.51;0.79	0.48 (<0.001)	0.30;0.65
NHQ	32 (36)	0.52 (<0.001)	0.34;0.69	0.37 (<0.001)	0.18;0.56
HQNO	26 (29)	0.62 (<0.001)	0.49;0.74	0.44 (<0.001)	0.26;0.62
NQNO	30 (34)	0.71 (<0.001)	0.61;0.82	0.44 (<0.001)	0.26;0.63
C7-PQS	10 (11)	0.45 (<0.001)	0.28;0.62	0.20 (0.081)	0.01;0.40
C9-PQS	9 (10)	0.43 (<0.001)	0.26;0.60	0.20 (0.091)	0.02;0.37

AQ=2-alkyl-4 quinolones; 95%CI; 95% confidence intervals, n= number; HHQ= 2-heptyl-4-hydroxyquinoline; NHQ= 2-nonyl-4-hydroxyquinoline; C7-PQS=2-heptyl-3-hydroxy-4(1*H*)-quinolone; C9-PQS= 2-nonyl-3-hydroxy-4(1*H*)-quinolone; HQNO= 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO= 2-nonyl-4-hydroxyquinoline-*N*-oxide, N; number of samples with AQ detected out of a total of 89 samples *;n=77; °; n=75, where n is the number of samples with data available for matched analysis.

4.3.2 Correlations between HHQ, NHQ and HQNO levels in saliva with quantitative load of P. aeruginosa in sputum

The quantitative load of *P. aeruginosa* in sputum was significantly correlated with 4 AQs: HHQ (r=0.477, *p*<0.001), NHQ (r=0.374, *p*=0.001), HQNO (r=0.443, *p*<0.001) and NQNO (r=0.441, *p*<0.001) concentrations in saliva (Table 4-3 and Figure 4-1). The remaining 2 AQs did not reach statistical significance with quantitative *P. aeruginosa* load: C7-PQS (r=0.2013, *p*=0.081) and C9-PQS (r=0.197, *p*=0.091).

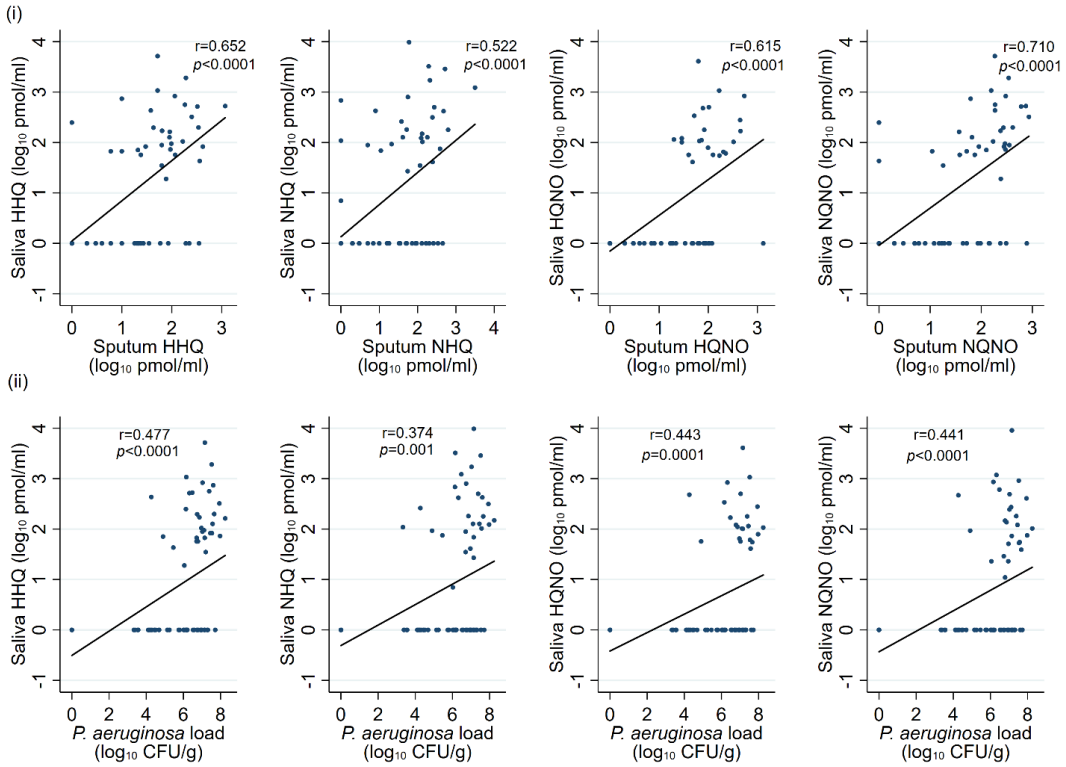

Figure 4-1. Spearman correlations between salivary concentrations of four AQs and (i) spontaneous sputum AQs concentrations and (ii) and *P. aeruginosa* load measured in sputum using PCR.

Figure 4-1 legend; Median (interquartile range) for sputum *P. aeruginosa* load by was 7.03 $\log_{10}$CFU/g (6.43 to 7.48) of sputum. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO: 2-nonyl-4-hydroxyquinoline-*N*-oxide, r= Spearman's correlation coefficient.

4.4 Discussion

This is the first study to examine saliva samples obtained from adults with CF for the presence of AQ quorum signal molecules produced by *P. aeruginosa*. All six AQs were detected in salivary samples and there were positive correlations between concentrations of AQs measured in saliva and corresponding sputum samples. In addition, salivary concentrations of four AQs (HHQ, NHQ, HQNO and NQNO) correlated significantly with quantitative load of sputum *P. aeruginosa* measured using PCR.

It is well known that *P. aeruginosa* is highly adapted to the lung environment and highly prevalent in the CF lung. However, diagnosing pulmonary *P. aeruginosa* can be difficult in people who are unable to spontaneously produce sputum. Furthermore, spontaneous sputum production is likely to decrease in the longer term due to the widespread use of highly effective cystic fibrosis transmembrane conductance regulator (CTFR) modulator therapies which have improved the disease trajectory [333]. However, despite restoration of the airway surface fluid with these personalised therapies, CFTR modulators do not significantly change bacterial diversity or *P. aeruginosa* load in the lung [334] and there is a clinical requirement for ongoing *P. aeruginosa* surveillance in the CF airways. Therefore, there is a need to develop accurate non-invasive methods to detect and quantify *P. aeruginosa* in CF without the need to use invasive techniques such as bronchoalveolar lavage.

Focusing on AQ detection in saliva for the early diagnosis of *P. aeruginosa* is biologically justified. The oral cavity is a potential reservoir for *P. aeruginosa* for initial colonisation which may precede chronic lung infection [335] and this is supported by data showing similarities between the oral and lung microbiota [336]. In addition, AQs upregulate virulence factors known to be important in establishing early infection and AQs have been detected in the systemic circulation of people with CF during early *P. aeruginosa* infection [337].

4.4.1 Strengths

Strengths of this study include a large multicentre cohort and the pre-treatment of sputum with PMA which enabled only viable *P. aeruginosa* bacterial cells to be amplified and quantified by PCR, providing a precise quantitative measure of the burden of infection.

4.4.2 Limitations

This study has some limitations that should be considered when interpreting these data. Firstly, this study is a proof of concept pilot study. It is unclear if AQs in the saliva are correlated with more severe disease in CF and therefore these findings

need to be validated, including with a control population of people with CF who have milder disease and do not have pulmonary *P. aeruginosa,* to accurately determine its potential use in the clinical setting. Secondly, participants rinsed their mouths with tap water prior to saliva sample collection and this may have adversely impacted the sensitivity of the analysis or led to false positive results due to potential water contamination. From our results, we cannot determine if the AQs detected in the saliva originate from the upper airway sources or from oral contamination from expectorated sputum from lower airways colonisation. AQs are also produced by a few other related bacterial species, including *Burkholderia cepacia* complex [280, 300, 315]. Participants known to have previously isolated *Burkholderia cepacia* complex were excluded from the study to minimise this risk. In addition, chronic *P. aeruginosa* infections in the CF lung can give rise to QS deficient mutants (such as LasR mutants) [338]. However, there has been no recorded evidence of AQ mutants in this setting to date [256, 339].

One limitation to the potential use of salivary AQs as an early non-invasive diagnostic assay for *P. aeruginosa* infection is that the sensitivity is only 44%. However, this is simply a proof of principle study demonstrating for the first time that salivary AQs can measure *P. aeruginosa* infection. Further studies refining the use of salivary AQs in the early diagnosis of *P. aeruginosa* infection can aim to increase the sensitivity of this approach; possibilities include the collection of three samples and avoidance of rinsing the mouth prior to saliva collection. In addition, refinement of the liquid chromatography-tandem mass spectrometry technology is needed to increase the sensitivity of detecting salivary AQs. Finally, further prospective studies with regular longitudinal sampling of salivary samples are needed to determine the variability of AQs *in vivo* and ensure both the accuracy and generalisability of these findings to the wider CF population as a whole.

4.4.3 Summary

In summary, *P. aeruginosa* QS signal molecules can be detected in the saliva obtained from adults with CF and chronic pulmonary *P. aeruginosa* infection. Salivary

AQ levels correlated significantly with both the concentrations of AQs and *P. aeruginosa* in the sputum. However, further refinement of the methodologies is needed to improve the sensitivity of detecting AQs in saliva to be clinically useful as non-invasive biomarkers for pulmonary *P. aeruginosa* in the future.

4.4.4 Author Contributions

I would like to acknowledge the below co-authors that contributed towards data interpretation, data presentation and writing of the manuscript which was accepted for publication in the Journal of Cystic Fibrosis in September 2021.

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Chapter 5. Viable anaerobic bacteria in the sputum microbiota are associated with increased decline in lung function in individuals with cystic fibrosis

5.1 Introduction

In this chapter we describe the first study to prospectively investigate viable anaerobic bacteria present in the sputum microbiota and their relationship with long-term lung function decline in adults with CF. We performed 16S rRNA analysis using a viability quantitative PCR technique on sputum samples obtained from a prospective cohort of 70 adults with CF and collected clinical data over an 8-year follow-up period. We examined the associations of the 10 most abundant obligate anaerobic bacteria present in the sputum with annual rate of FEV_1 change.

5.1.1 Background

Chronic pulmonary infection and recurrent pulmonary exacerbations are associated with both increased morbidity and mortality in cystic fibrosis (CF) [313]. Historically, conventional microbiological culture techniques have routinely isolated several distinct aerobic species, such as *Staphylococcus aureus, Haemophilus influenzae* and *P. aeruginosa,* which have been extensively studied [340].

However, conventional culture techniques only identify a very small proportion of the bacteria present in the lung environment. In recent years, culture-independent techniques such as polymerase chain reaction (PCR) and microbiome analysis have shown that the CF airways are complex polymicrobial environments and often contain anaerobic bacteria [194-196], which are not routinely cultured in clinical practice.

The CF airways contain mucus which adheres to epithelial surfaces. Within these mucus plugs, are biofilms with steep oxygen gradients which are considered to be hypoxic environments, providing a niche for anaerobic bacteria [341, 342]. However, it is not currently known whether these anaerobic bacteria play a pathophysiological role in lung damage, pulmonary exacerbations or long-term adverse outcomes in this patient population [341].

Sputum microbiome studies in CF have shown that the composition of bacterial communities remains relatively stable during exacerbations despite systemic antibiotic therapy [140, 201], and loss of species diversity is associated with reduced lung function [140, 198]. However, there are limited longitudinal data on the relationship between the sputum microbiome and long-term clinical outcomes [343]. Furthermore, whilst photoreactive dyes have been increasingly used to amplify and quantify only the bacterial cells with intact cell membranes in 16S rRNA sequencing [316], previous sputum microbiome studies in CF have not differentiated between viable and dead bacteria.

5.1.2 Aims

Using this viable cell technique, we investigated whether the presence and relative abundance of the ten most abundant, viable, obligate anaerobic bacterial species in CF sputum were associated with accelerated lung function decline in an eight-year follow up period.

5.2 Methods

5.2.1 Participants and study design

We analysed sputum samples obtained adults with CF who had previously participated in a biomarker study, the full details of which have been published [280]. In summary, participants were recruited at clinical stability from two UK adult CF centres between the years 2009 and 2011. Adults with CF were clinically stable at the study visit, having not experienced a pulmonary exacerbation requiring intravenous

(IV) antibiotics in the preceding 4 weeks Baseline demographic data were collected, and sputum plugs were stored for future microbiome analysis.

5.2.2 Clinical data

Lung function data were obtained from the UK CF registry [344] using the highest recorded forced expiratory volume in 1 second (FEV_1) of the preceding year. Annual data on lung function were collected from the participants from the year of recruitment to the end of the study period in 2017 or until year of death or lung transplantation.

5.2.3 Sample processing and microbiome analysis

Sputum plugs were harvested for microbiome analysis with an equal volume of 0.9% saline and stored in -80°C freezers. Prior to DNA extraction, known weight of sputum aliquots (up to 0.2 g) were pre-treated with propidium monoazide (PMA). PMA is unable to penetrate intact cell membranes and therefore only binds to the DNA of cells with compromised cell membranes [332]. Photo-activation of PMA results in covalent DNA modification and damage. This subsequently prevents amplification via polymerase chain reaction (PCR). Non-PMA treated matching sputum samples were used as positive controls.

DNA extraction was conducted using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich Co. Ltd., Dorset, UK) according to manufacturer's instructions, incorporated with additional enzymatic and physical disruption of samples. DNA was resuspended in 50 µL of Elution Solution and quantified using a Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK). Phosphate-buffered solution was used as negative controls in DNA extraction and library preparation for sequencing and no amplification was detected above the lowest limit of detection (10 CFU/µl). Paired-end (300bp) sequencing of 16S rRNA V3-V4 region for 75 DNA samples were then performed on the MiSeq platform (Illumina, USA). Sequence processing and analysis included quality filtering of reads, chimera removal and assignment to Operational Taxonomic Units (OTUs) via the C9VLC pipeline and the QIIME software package (version 1.9.1, http://qiime.org/) (Eurofins, Germany).

5.2.4 Statistical analysis

Yearly change in lung function was predicted for each individual using linear regression models of CF registry-recorded FEV_1 values with their corresponding dates, and is reported as FEV_1 mL change per year (mL/yr).

The relative abundance of each Operational Taxonomic Unit (OTU) was initially expressed as a percentage (%) of the total number of reads in each individual. To allow comparison across the cohort, the % OTUs were transformed to Colony Forming Unit (CFU/g) of sputum by multiplying the % values with live total bacterial load in each sample accordingly, as informed by quantitative polymerase chain reaction (qPCR) [345]. The relative abundance (CFU/g of sputum) was subsequently transformed after the addition of 1 (log_2) for statistical analyses.

The ten most abundant anaerobic OTUs were determined by ranking the relative abundance of the anaerobic OTUs present in all participants. Obligate anaerobe OTUs were identified based on literature [346, 347] and culture collections databases (NCTC, ATCC and DSMZ). Only the taxonomic level of species were included in the analyses. Generalised linear regression models were used to analyse presence or absence and relative abundance (log_2) on annual rate of FEV_1 change.

All analyses were performed in Stata SE15 statistical software (Texas, USA), a p-value<0.005 was considered statistically significant following Bonferroni correction for multiple comparison of ten OTUs.

5.3 Results

Sputum samples and data were available for 70 (93.1%) of the 75 participants in the original study. The relative abundance of the anaerobic OTUs present in all participants are summarised in Table 5-1.

Sixty-eight (97.1%) of participants were chronically colonised with *P. aeruginosa* [169]. Baseline clinical characteristics and OTU abundance are summarised in Table 5-2.

A total of 1.2 million sequencing reads were obtained from the pooled sputum of each participant (n=70) and assigned to 212 OTUs. The 212 OTUs assignment was determined by best-matching reference sequences to the most specific (lowest) taxonomic level. Within this dataset, 121 OTUs were classified as facultative anaerobes or aerobes, 73 OTUs as obligate anaerobes, while 18 OTUs assigned to taxa above genera were categorised as 'unclassified'. The lung microbiome for each CF individual varied from 5 to 44 different OTUs, with the highest richness of obligate anaerobes observed at 17 OTUs per participant (Table 5-2). Obligate anaerobes were present in 65/70 CF individuals, ranging from 0.1% to 70.6% of the total microbiome (Figure 5-1). The 10 most prevalent obligate anaerobes are summarised in Table 5-3.

Table 5-1. The summary of anaerobic Operational Taxonomic Units.

	Operational Taxonomic Unit (OTU)	N	Prevalence %	Cumulative species abundance (log_2 CFU/g of sputum)
Obligate anaerobes	*Prevotella melaninogenica*	52	74.3	23.52
	Prevotella sp.	43	61.4	23.39
	Scardovia wiggsiae	31	44.3	21.13
	Alloprevotella Prevotella sp. oral taxon 472	22	31.4	19.81
	Porphyromonas pasteri	21	30.0	20.07
	Porphyromonas sp.	17	24.3	20.42
	Parvimonas micra	15	21.4	18.42
	Veillonella sp.	15	21.4	18.38
	Peptostreptococcus sp.	14	20.0	19.54
	Prevotella pallens	14	20.0	19.10
	Catonella sp.	11	15.7	17.87
	Atopobium sp.	10	14.3	19.83
	Fusobacterium nucleatum	9	12.9	18.04
	Mogibacterium sp.	9	12.9	16.44
	Prevotella nanceiensis	9	12.9	18.33
	Stomatobaculum longum	9	12.9	20.07
	Prevotella oris	8	11.4	21.47
	Atopobium rimae	7	10.0	20.07
	Ihubacter sp.	7	10.0	18.68
	Alloprevotella tannerae	6	8.6	16.14
	Porphyromonas endodontalis	6	8.6	17.75
	Prevotella oulorum	6	8.6	19.66
	Oribacterium sp.	5	7.1	17.75
	Peptoniphilus sp.	5	7.1	15.99
	Porphyromonas catoniae	5	7.1	16.05
	Prevotella denticola	5	7.1	18.50
	Finegoldia sp.	4	5.7	14.14
	Porphyromonas sp. C1075	4	5.7	16.43
	Prevotella histicola	4	5.7	17.29
	Tannerella forsythia	4	5.7	14.21
	Tannerella sp.	4	5.7	16.03
	Tannerella sp. oral taxon HOT-286	4	5.7	14.97
	Anaerococcus sp.	3	4.3	12.87
	Fusobacterium periodonticum	3	4.3	12.64
	Moryella indoligenes	3	4.3	19.76
	Shuttleworthia sp.	3	4.3	15.87
	Bifidobacterium sp.	2	2.9	20.31
	Dialister sp.	2	2.9	15.69
	Olsenella sp.	2	2.9	13.67
	Oribacterium parvum	2	2.9	11.81
	Peptostreptococcus anaerobius	2	2.9	16.38
	Prevotella nigrescens	2	2.9	15.10

	Prevotella salivae	2	2.9	13.67
	Solobacterium moorei	2	2.9	11.17
	Anaerococcus obesiensis	1	1.4	11.23
	Atopobium sp. DMCT15023	1	1.4	17.61
	Bacteroides fragilis	1	1.4	11.40
	Bifidobacterium breve	1	1.4	11.23
	Catonella morbi	1	1.4	12.53
	Fusobacterium necrophorum	1	1.4	12.81
	Johnsonella sp. oral taxon 166	1	1.4	14.82
	Lachnoanaerobaculum umeaense	1	1.4	16.61
	Megasphaera sp. sp4-iso-1H02x2	1	1.4	15.25
	Parvimonas sp. KA00067	1	1.4	8.88
	Peptococcus sp. oral taxon 167	1	1.4	12.97
	Porphyromonas gingivalis	1	1.4	9.97
	Prevotella conceptionensis	1	1.4	13.77
	Prevotella intermedia	1	1.4	14.05
	Prevotella shahii	1	1.4	12.26
	Prevotella sp. 8404125	1	1.4	10.89
	Prevotella sp. oral taxon 292	1	1.4	14.55
	Prevotella sp. oral taxon 299	1	1.4	11.29
	Ruminiclostridium cellobioparum	1	1.4	11.60
	Scardovia sp.	1	1.4	13.43
	Shuttleworthia sp. MSX8B	1	1.4	12.00
	Slackia sp. CM382	1	1.4	12.97
	Sneathia amnii	1	1.4	10.10
	Sneathia sanguinegens	1	1.4	11.23
	Treponema lecithinolyticum	1	1.4	15.67
	Treponema maltophilum	1	1.4	12.73
	Treponema sp. OMZ 838	1	1.4	9.88
	Veillonella atypica	1	1.4	15.18
	Veillonella parvula	1	1.4	11.89

N; number of individuals with OTU present.

Table 5-2. Baseline clinical characteristics, summary of clinical data during follow up period and OTU abundance in each participant.

Variable	Baseline (n=70)	
Nottingham University Hospitals NHS trust	38	
University Hospitals Birmingham NHS Foundation Trust	32	
Age in years: median (range)	30.6 (17.8 to 61.5)	
Gender, males (%)	46 (65.1)	
FEV_1 % predicted: mean (SD)	58 (±20)	
Absolute FEV_1 in L: mean (SD)	2.2 (±0.9)	
BMI in kg/m^2: mean (SD)	22.9 (± 3.4)	
P. aeruginosa status at baseline: n (%)		
Never	0 (0)	
Free	1 (1.4)	
Intermittent	1 (1.4)	
Chronic	68 (97.1)	
Variable	N (%)	Outcome
Follow up time¥ (years)	70 (100)	6.8 (6.2-8.1)
Number of lung transplantation	8 (11.4)	
Number of deaths	10 (14.2)	
Rate of lung function change per year§:		
Absolute FEV_1 (ml)	68 (97)	-53.2 (±71.4)
Percent predicted FEV_1 (%)	68 (97)	-1.4 (±1.9)
Variable per participant (n=70)	Range	Mean (SD)
Number of reads	9748 - 48,504	17,458.9 (±6306.1)
Number of OTUs	5 - 44	22.7 (±9.6)
Number of obligate anaerobe OTUs	0 - 17	6.3 (±4.4)
Total abundance, CFU/g of sputum	$5.0 \times 10^4 – 3.9 \times 10^7$	3.5×10^6 (6.0×10^6)
Obligate anaerobe abundance, CFU/g of sputum	$0 – 7.6 \times 10^6$	6.1×10^5 (1.3×10^6)

n = number of participants with data available; SD = standard deviation, *P. aeruginosa* status of participants defined by Leeds criteria [169].
¥: reported as median and interquartile range. § reported as mean and standard deviation. N= number of participants with data available.

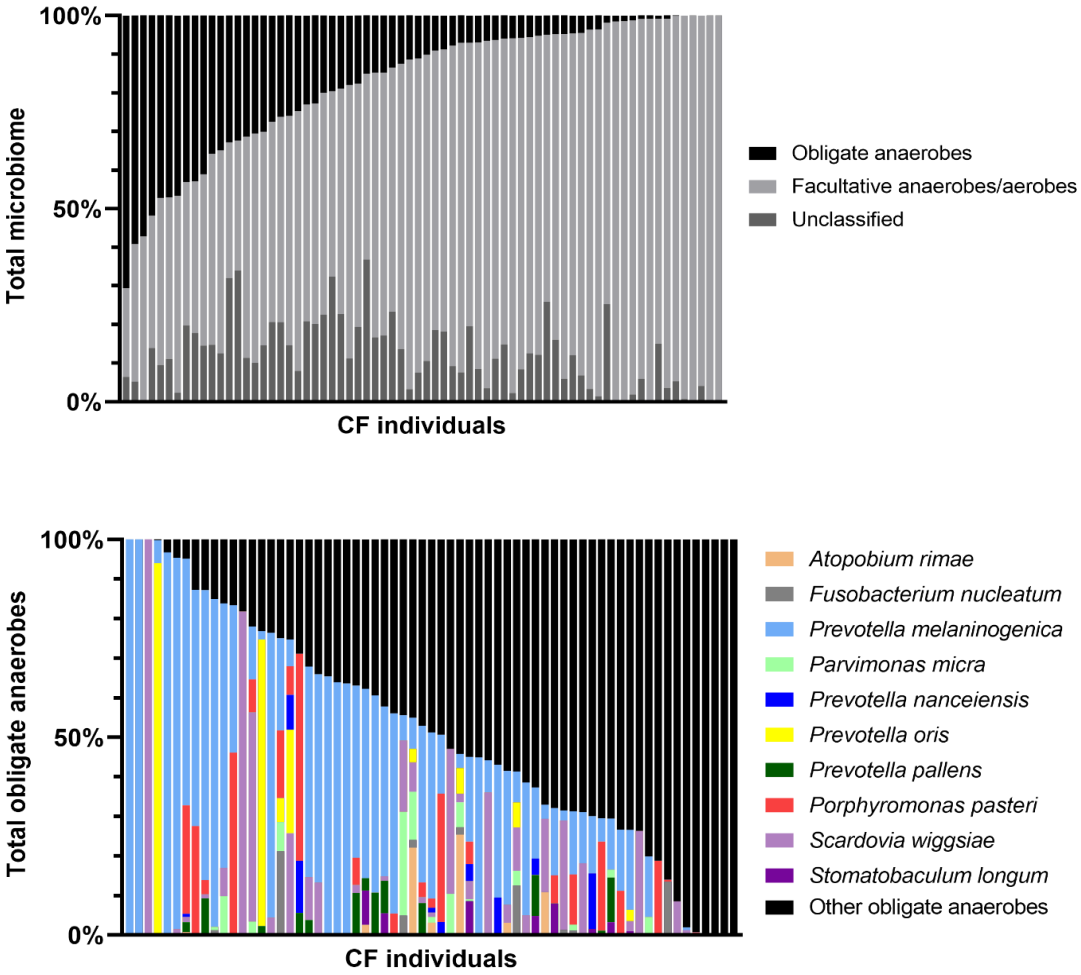

Figure 5-1. The composition of (A) total obligate anaerobe OTUs within the viable CF total microbiome at clinical stability (n=70) and (B) individual OTUs included in the analysis within the total obligate anaerobe group (n=65).

Table 5-3. The 10 most abundant obligate anaerobic bacteria identified at species level present in sputum at clinical stability (N=70).

Anaerobic species (OTUs)	n (%)	Range*
Prevotella melaninogenica	52 (74.3)	0 to 3.7 x 10^6
Scardovia wiggsiae	31 (44.3)	0 to 5.8 x 10^5
Porphyromonas pasteri	21 (30.0)	0 to 2.6 x 10^5
Parvimonas micra	15 (21.4)	0 to 2.5 x 10^5
Prevotella pallens	14 (20.0)	0 to 2.4 x 10^5
Stomatobaculum longum	9 (12.9)	0 to 6.6 x 10^5
Fusobacterium nucleatum	9 (12.9)	0 to 1.5 x 10^5
Prevotella nanceiensis	9 (12.9)	0 to 2.0 x 10^5
Prevotella oris	8 (11.4)	0 to 1.6 x 10^6
Atopobium rimae	7 (10)	0 to 4.5 x 10^5
n; number of participants in which the individual bacterial species was present *; relative abundance before log_2 transformation		

5.3.1 The impact of the ten most abundant anaerobic species present in sputum on annual rate of FEV_1 change over an 8 year follow-up period

Two of the 10 anaerobic species studied were associated with lung function decline: *Porphyromonas pasteri* and *Prevotella nanceiensis* (Table 5-4). The effect size of annual rate of FEV_1 decline if these viable organisms were present compared to absent was -52.3mL/yr (95% CI:-87.7 to -16.9, *p*=0.004) and -67.9mL/yr (95%CI: -115.6 to -20.1, *p*=0.005 (Table 5-4), respectively. Similarly, the relative abundance of live *P. pasteri* and *P. nanceiensis* was associated with a greater annual rate of FEV_1 decline of -3.7mL/yr (95% CI: -6.1 to -1.3, *p*=0.003) and -5.3mL/yr (95% CI: -8.7 to -1.9, *p*=0.002; Table 5-4) for each log_2 increment of abundance, respectively.

Table 5-4. The effect size of presence relative to absence and relative abundance of the 10 most abundant obligate anaerobic species on annual rate of FEV_1 (mL) decline, N=68.

Anaerobic species	N(%)	Coefficient (β)±	95% CI±	Coefficient (β)*	95%CI*
Prevotella melaninogenica	50 (73.5)	24.7	-13.6; 63.0	0.4	-2.0; 2.8
Scardovia wiggsiae	15 (22.1)	-7.6	-43.0; 27.8	-1.8	-4.1; 0.5
Porphyromonas pasteri	9 (13.2)	-52.3	**-87.7; -16.9**	-3.7	**-6.1; -1.3**
Parvimonas micra	7 (10.3)	6.5	-34.7; 47.7	-0.7	-4.1; 2.7
Prevotella pallens	20 (29.4)	14.4	56.5; 27.8	-0.7	-3.8; 2.4
Stomatobaculum longum	9 (13.2)	11.8	-38.6; 62.2	-0.7	-4.4; 2.8
Fusobacterium nucleatum	8 (11.8)	35.4	14.3; 85.2	2.3	-1.7; 6.2
Prevotella nanceiensis	31 (45.6)	-67.9	**-115.6; -20.1**	-5.3	**-8.7; -1.9**
Prevotella oris	14 (20.6)	-11.2	-64.2; 41.8	-1.0	-4.4; 2.4
Atopobium rimae	9 (13.2)	-22.6	-78.6; 33.4	-2.0	-5.5; 1.5

N: number of bacteria present in sputum of participants, β: coefficient of annualised rate of FEV_1 decline (mL/yr) ±: Effect size of annual rate of FEV_1 decline if bacterial species is present relative to absent, *: log_2 relative abundance and annual rate of FEV_1 decline, 95% CI; Confidence intervals. 95% CI in bold are considered significant after Bonferroni correction (p=0.005)

5.4 Discussion

This is the first study to prospectively investigate viable anaerobic bacteria present in the sputum microbiota and relationship to long-term outcomes in adults with CF. The presence and relative abundance of both *Porphyromonas pasteri* and *Prevotella nanceiensis* in a cohort predominately colonised with *Pseudomonas aeruginosa* were associated with a greater annual lung function decline over the 8-year study period.

The lower airways in CF are polymicrobial and often contain anaerobes [194-196]. These anaerobes may be the result of repeated micro aspirations of oral flora in combination with an abnormal mucociliary clearance mechanism in CF airways [348], although not all data support this hypothesis [349-351]. Despite the high abundance of anaerobes in the CF lower airways, their pathogenicity in the CF airways is not well understood.

There is much deliberation over whether anaerobic bacteria, such as *Prevotella* sp. which are known to be pathogenic and to harbour antimicrobial resistance genes [352], can also interact with other bacterial pathogens, such as *P. aeruginosa,* to enhance their virulence [353]. *Prevotella* species can protect *P. aeruginosa* against the activity of ceftazidime, an anti-pseudomonal antibiotic commonly used to treat pulmonary exacerbations [354]. Our data support these observations, suggesting that *Prevotella* sp. may be associated with adverse clinical outcomes. However, our observation that *Porphyromonas* was associated with adverse clinical outcomes is not supported by other studies. Studies in children with CF found that *Porphyromonas* sp. may protect against *P. aeruginosa* colonisation [355, 356] and *Porphyromonas* sp. is also less abundant during pulmonary exacerbations, thus suggesting a protective role [355]. However, these studies were both performed in children without *P. aeruginosa,* while our study was performed in adults with chronic pulmonary *P. aeruginosa* and more severe pulmonary disease.

We suggest that changes in community dynamics over time, spatial heterogeneity of the lungs and differing microenvironments may account for the contrasting observations. Zemanick *et al* found that the presence of sputum anaerobes during a pulmonary exacerbation were associated with improved lung function and less inflammation [199], although there was significant variability in anaerobes in response to antibiotics (15). This suggests that diverse anaerobic species present in the airway may have distinct pathogenic or protective roles depending on individual interactions in the lung microbiota [343, 357].

5.4.1 Strengths

Strengths of the study include the multicentre study population who were recruited from two adult specialist CF centres. Longitudinal follow up over an 8-year period provided robust linear modelling of lung function to predict FEV_1 outcomes. In addition, by using a novel PMA pre-treatment, we were able to give a more accurate estimate of viable anaerobic bacteria.

5.4.2 Limitations

There are a number of limitations in this study that should be considered when interpreting these data. The enumeration of bacterial cells using the viability PCR technique has not been validated in comparison to standard culture methods for CF sputum [332]. Sputum samples were taken once at baseline only and compared with adverse clinical outcomes therefore findings should be considered as hypothesis generating. Whilst this offers novel insights, longitudinal profiling of the sputum microbiota over multiple time points would aid our understanding further on the microbiological anaerobic community dynamics.

5.4.3 Summary

Our findings support a potential association of specific *Prevotella* and *Porphyromonas* species abundantly present in CF sputum with long-term lung function decline. These findings suggest that certain anaerobic bacteria may contribute to lung disease and CF pathophysiology which should be confirmed with a large, prospective cohort with regular microbiome profiling combined with enhanced sequencing strategies.

5.4.4 Future studies

As this chapter describes a hypothesis generating study, further studies are needed. A large prospective observational cohort study with regular longitudinal sampling is needed to fully explore changes in the anaerobic lung microbiota over time and lung function decline in CF.

5.4.5 Author Contributions

I would like to acknowledge the below co-authors that contributed towards data interpretation, data presentation and writing of the manuscript which was submitted for publication in the Journal of Medical Microbiology, June 2021.

Nur Masirah M. Zain, Iain Stewart, Andrew Fogarty, Edward F. Nash, Joanna L Whitehouse, Alan R. Smyth, Andrew K. Lilley, Alan Knox, Paul Williams, Miguel Cámara, Kenneth Bruce and Helen L. Barr.

Chapter 6. Methodology and study design: Airway infection patients treated for an exacerbation of bronchiectasis; a feasibility study

6.1 Introduction

This chapter describes the methodology of a cohort observational study of adults with non-CF bronchiectasis who required intravenous antibiotics for a pulmonary exacerbation.

6.2 Background

Non- CF bronchiectasis is a relatively neglected disease that causes substantial morbidity to patients and is associated with increased mortality. It is characterised by structural damage to the lungs, often caused by infection in childhood. It results in recurrent chest infections that are difficult to treat with oral antibiotics as a consequence of a combination of the microbes involved and antibiotic resistance, necessitating admission to hospital for intravenous or inhaled antibiotics. Pulmonary exacerbations and chronic infection are a huge healthcare burden in this patient cohort, with implications for both the individual patient and society as a whole. Furthermore, little is known about the relationship between the different phenotypes and genotypes of these pathogens, coupled with lung microbiome and QS signal molecule data.

6.3 Rationale

Selection of antibiotics is often empirical, and the care of these patients would be improved with better understanding of the microbiological organisms that cause these infections, and biomarkers to guide clinical decisions. In particular, the heterogeneity

of conditions in non- CF bronchiectasis need a greater understanding both into drivers of pathogenicity and the appropriate diagnostic and management strategies.

6.4 Overall aims:

- To assess the feasibility of collecting and analysing *P. aeruginosa* quorum sensing molecules in a non-CF bronchiectasis cohort.
- To establish a cohort of patients with non-CF bronchiectasis and determine both clinical and microbiological factors (including culture and culture-independent methods) that may influence disease severity, pulmonary exacerbations and mortality.

COVID-19 IMPACT STATEMENT

Due to the COVID-19 pandemic, the non-CF bronchiectasis feasibility study for quorum sensing molecules was delayed by the time of writing this thesis. As such, further analysis was performed to explore the role of novel urinary inflammatory molecules and pro-inflammatory cytokines in this cohort.

6.5 Approval for the study

The research ethics committee (18/WM/0125) and the research and development department of Nottingham University Hospital Trust (17RM028) gave approval for the study.

6.6 Study design

This was a single centre prospective observational study based at Nottingham University Hospitals. Participants in the study were consented to be seen at 3 time points; within 72 hours of a pulmonary exacerbation, within 72 hours after an intravenous antibiotic course and at clinical stability (4-6 weeks after intravenous antibiotic therapy).

All patients who were recruited at pulmonary exacerbation were either an inpatient in Nottingham City Hospital or referred for outpatient parenteral antibiotic therapy (OPAT). Both the choice of antimicrobial therapy administered and the course duration were at the discretion of the clinician.

At every visit, spontaneous sputum, blood and random urine samples were collected. Pulmonary function tests were performed with a calibrated spirometer according to the joint ERS/ATS criteria [358]. In addition, participants were required to fill out a validated quality of life questionnaire (Qol-B) at every visit [61]. The hospital and electronic records were reviewed for information including the aetiology of bronchiectasis, co-morbidities, microbiological data and concurrent medications. The severity of bronchiectasis for the participants were assessed using the bronchiectasis severity index (BSI) [6] and bronchiectasis aetiology comorbidity index (BACI) [67]. Using these scores, the cohort was stratified into mild, moderate and severe disease. The criteria for chronic colonisation of a microbiological pathogen was defined by the isolation of potentially pathogenic bacteria in sputum culture on two or more occasions, at least 3 months apart in a 1-year period (4).

The medical electronic records of the participants were reviewed after the end date of IV antibiotics in the primary recruitment episode until the end of the study period on 30/11/2020. The data explored included; time to next exacerbation (measured as the interval between the date of completion of IV antibiotics and the date of commencement of IV antibiotics for next pulmonary exacerbation), and mortality.

6.6.1 Sample size

A maximum of 50 patients who were experiencing a pulmonary exacerbation and requiring administration of intravenous antibiotics were recruited. As this was a feasibility study, the sample size was based on a conservative estimate of 20% reduction in HHQ from our pilot study in the CF population, using a 95% CI and a power of 80% which indicates a sample size of 40 to detect a significant difference

6.6.2 Pulmonary exacerbation criteria definition

The definition of a pulmonary exacerbation of bronchiectasis was defined according to Hill *et al* [69], which is a consensus definition for clinical trials:

A deterioration in three or more of the following key symptoms *for at least 48 hours:*

- cough
- sputum volume and / or consistency
- sputum purulence
- breathlessness and / or exercise tolerance
- fatigue and / or malaise
- haemoptysis

AND a clinician determines that a change in bronchiectasis treatment is required.

6.6.3 Recruitment

Patients who were eligible and admitted as an inpatient or receiving outpatient antibiotic treatment (OPAT) at Nottingham City Hospital were invited to participate in the study. Participants who completed the IV antibiotic course at home through OPAT returned to hospital for a review by the research and clinical team on completion of the course.

6.6.4 Inclusion criteria

- Diagnosis of bronchiectasis
- Age 16 years or over
- Patients were experiencing a pulmonary exacerbation; defined by Hill *et al* [69] and the clinician felt IV antibiotics were the required treatment.

6.6.5 Exclusion criteria

- The participants were receiving terminal care
- The participant was unable to provide consent

6.6.6 Study timetable

1. Screening of patients by the researcher
2. Patients contacted by the researcher if inclusion criteria fulfilled
3. Informed consent
4. Baseline visit- at the start of IV antibiotics (within 72 hours)
5. Second visit- at the end of IV antibiotics (within 72 hours)
6. Third visit- 4-6 weeks post exacerbation (or when the patient had not received IV antibiotics for at least 4 weeks at a time point after the second visit)
7. Medical notes and electronic records reviewed up until the end of November 2020

6.6.7 Pulmonary function testing

Spirometry was performed using a portable spirometer (MIR Spirolab) which was compliant with ERS/ATS standards [358]. A single use disposable (FlowMIR) that had been individually factory calibrated with a pre-connected mouthpiece, was used for each participant visit. The contraindications for spirometry were as follows:

- Active or suspected pulmonary *Mycobacterium tuberculosis* infection
- Active haemoptysis

6.7 Sampling and Processing

Spontaneous sputum samples, blood and urine samples were planned to be taken within 72 hours of both commencing and completing IV antibiotics for an acute pulmonary exacerbation. The 72 hour interval was agreed to facilitate maximum recruitment of participants in a hospital and outpatient setting. Further samples were taken 4-6 weeks post pulmonary exacerbation at clinical stability (or where the participant had not been administered IV antibiotics for at least 4 weeks after the second visit).

All samples were collected in sterile containers, transferred to the research laboratory on wet ice in a sealed polystyrene box and intended to be processed and stored within 2 hours. All samples were processed in an airstream Class ll biological safety cabinet according to the manufacturer's guidelines.

All samples were initially collected, processed and stored at the *Respiratory Biomedical Research Centre (BRC), Clinical Sciences Building, University of Nottingham.* Initially, sputum, blood and urine samples were sent for QS analysis at *The Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham, UK.* In addition, further supplementary llaboratory analysis was performed for urinary inflammatory biomarkers (discussed in Chapter 8) and pro-inflammatory cytokines (discussed in Chapter 9). In addition, sputum samples were sent for quantitative polymerase chain reaction (PCR) at *The Institute of Pharmaceutical Science, King's College London, London, UK,* (discussed in Chapter 7).

6.7.1 Blood and urine processing

At each visit, 8ml of venous blood was collected in 2x 4ml pre-cooled EDTA blood tubes. Venous blood samples were centrifuged at 1000 g for 15 min at 4°C, plasma was then separated into 6 aliquots of 500µl and snap frozen in either liquid nitrogen or dry ice (if liquid nitrogen was not available).

A 25mL 'random catch' urine sample was collected. This was then separated into 6 aliquots of 1mL and snap frozen in dry ice. These samples were then transferred, stored in -80 °C freezers and later thawed for subsequent analysis as described in Chapters 8 and 9.

6.7.2 Sputum sampling

All sputum samples were collected in sterile containers that were pre-cooled and stored in wet ice until processing. When possible, if not previously sent by the clinical team, a sputum sample was additionally sent to the hospital laboratory (QMC NHS laboratory) for routine microbiological culture and sensitivity testing.

6.7.3 Sputum processing

The sputum samples were processed in the BRC research laboratory. Sputum plugs were harvested and split for QS and quantitative PCR/microbiome analysis, quantitative microbiology (cultured on blood and pseudomonas isolation agar), and slide/supernatant processing for differential cell counts. The sputum supernatants were stored in cryotubes at -80° C for future studies.

6.7.4 Sputum plug harvesting

1. Empty the sputum sample into a sterile petri dish, using fine forceps isolate sputum plugs (solid or dense looking material) from clear salivary fluid using curved forceps and transfer onto petri dish lid.
2. Use forceps to gather sputum plugs into a single mass; move the mass around the lid in a circular motion to encourage saliva condensation and removal.
3. Transfer the purified sputum to the pre-weighed sterile petri dish and weigh the entire sputum specimen.
4. Divide the sputum into 3 and transfer into pre-weighed 15ml falcon tubes labelled S1 (supernatant and differential cell counts), QS (QS analysis) and M (quantitative microbiology). Re-weigh to determine the weight of sputum isolated in each tube.

6.7.5 Quantitative microbiology by culture

The sputum plug is mixed 1:1 with 0.1% dithiothreitol (DTT) in a sterile falcon tube, agitated with a sterile pipette and vortexed until a homogenous suspension is achieved.

1. Transfer 100µl into 400µl of 0.9% saline to give 10^{-1} concentration.
2. Subsequently transfer 100µl of 10^{-1} into 900µl 0.9% saline to give 10^{-2} concentration. Repeat twice more to obtain 10^{-3} and 10^{-4} concentrations.

3. 10^{-5} and 10^{-6} concentrations are obtained if processing occurred prior to the weekend.
4. Pipette 100µl of 10^{-2} onto both 1x blood agar plate (Columbia Agar Base, E&O Laboratories Ltd, UK) and 1x pseudomonas isolation agar plate (Pseudomonas Cetrimide Agar, Oxoid Ltd, UK). Sterile plate spreaders are then used to evenly distribute the solution across the plate. The plate is rotated and a sweeping motion is performed from the centre to the edges.
5. Repeat with 10^{-4} concentration.
6. Place inoculated plates into a Stuart S160 incubator (Bibby, Scientific, UK) at 37°C.
7. Count the number of colonies using a Stuart Colony Counter SC6 (Bibby, Scientific, UK) at 24 hours and 48 hours. After 48 hours, continue counting colonies until no further growth occurs or colonies are uncountable to a maximum of 5 days.
8. To calculate the colony forming units (CFU)/ml: multiply number of colonies observed by 10 and then again by dilution factor.

6.7.6 Quantitative microbiology by PCR

- The sputum plug is mixed 1:1 with 0.9% saline, agitated with a Pasteur pipette and vortexed for 3 minutes.
- Sputum solution is then distributed equally across 5 barcode labelled cryovials (minimum 50-100µl per aliquot). It was then kept on ice until transfer to -80˚C freezers.
- Sputum samples were sent on dry ice to The Institute of Pharmaceutical Science, King's College London, London, UK for quantitative PCR analysis.
- Prior to DNA extraction, sputum samples were pre-treated with propidium monoazide (PMA) to penetrate dead/compromised bacterial cells and bind to DNA as described previously by Rogers *et al* [332].

- PMA (20mM in water; Biotium, USA) was added to the samples to a final concentration of 50 µM followed by incubation in the dark on a rotating shaker for 30 min.
- The dye was then fixed to DNA by 15 min exposure to LED blue light (IB-Applied Science, Spain). Cells were pelleted at 10000 x g for 5 min prior to DNA extraction.
- DNA extraction was conducted using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich Co. Ltd., Dorset, UK) with the following modifications:
    - Samples were initially mixed with lysozyme (200 µL; 45 mg/mL, Sigma-Aldrich Co. Ltd., Dorset, UK) suspended in Gram-Positive Lysis Solution (included in the kit) followed by insertion of glass beads.
    - Cell disruption was then achieved by agitation in a Fastprep-24 Instrument (MP Biomedicals Europe, Illkirch, France) at 6.5 m/s for 60 sec twice and subsequently incubated at 37°C for 30 min.
    - Further steps remained unchanged, and the DNA was resuspended in 50 µL of Elution Solution (included in the kit).
- DNA concentrations were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK). *P. aeruginosa*-specific qPCR assay was performed as described previously [318].
- The lower limit of quantification (LLOQ) for *P. aeruginosa* in this assay was 100 CFU/g of sputum aliquot. Quantitative values were generated by Rotor Gene Q-series software (Qiagen, Crawley, UK) and expressed in CFU/g of sputum aliquot.

6.7.7 Supernatant/slide processing

The supernatant, cytospins for differential cell counts and quantitative microbiology analysis incorporated techniques from the Leicester respiratory group [359] and are summarised in detail below:

- Mix sputum plugs with Dulbecco's phosphate-buffered saline (D-PBS) to provide 8 volumes (ml) x sputum weight (g) (8ml/g sputum).
- Vortex sample for 15 seconds and rock on a bench Spiromix for 10 minutes on ice.
- Centrifuge at 600g for 10 minutes at 4°C.
- 6 volumes of the supernatant (6 *volumes of PBS added in previous step*) are then removed into a fresh 15ml falcon labelled *'PBS Supernatant'* and centrifuged at 1500g for 10 minutes at 4°C. The resulting supernatant is then split between six cryovials labelled '*PBS Supernatant'* and stored at -80°C.
- Once 6 volumes are removed, add two volumes of PBS (2 x original selected sputum weight) to the remaining sputum pellet followed by four volumes (4 x original selected sputum weight) of 0.2% DTT to the sputum sample (*equal volumes to that of supernatant removed in previous step*).
- Vortex sample for 15 seconds and rock on ice for 10 minutes.
- Filter the sample through a pre-wet D-PBS 48 µm gauze placed in a funnel and pre-weighed 15ml falcon, labelled S2. Re-weigh to determine the weight of sample.

6.7.8 Calculating viable cell count

Assess total cell count viability and level of squamous contamination using a Neubauer haemocytometer and the trypan blue exclusion method:

- Remove 10µl sample and mix with 10µl Trypan blue. Flood a chamber of the haemocytometer with 10µl of the cell suspension/Trypan blue solution and perform a cell count within 5 minutes.
- Count all cells in 9 fields of the haemocytometer (at least 100 cells), Figure 1. The cells are classified as viable leukocytes (unstained), dead leukocytes (blue) and squamous (whether viable or not). Cells touching the top and left lines are counted. Cells touching the bottom and right lines are not, Figure 2.

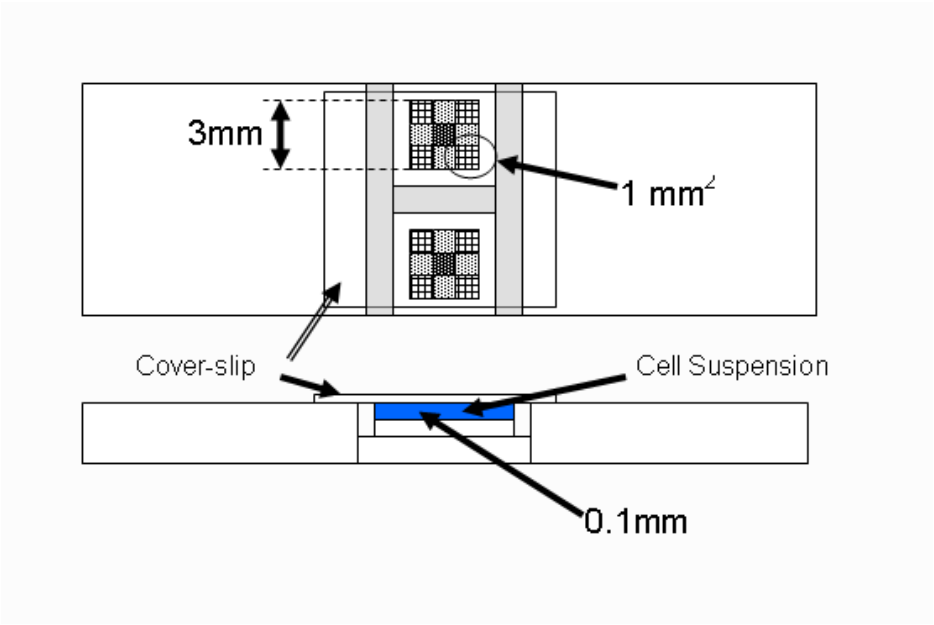

Figure 6-1. Haemocytometer dimensions and arrangement [360].

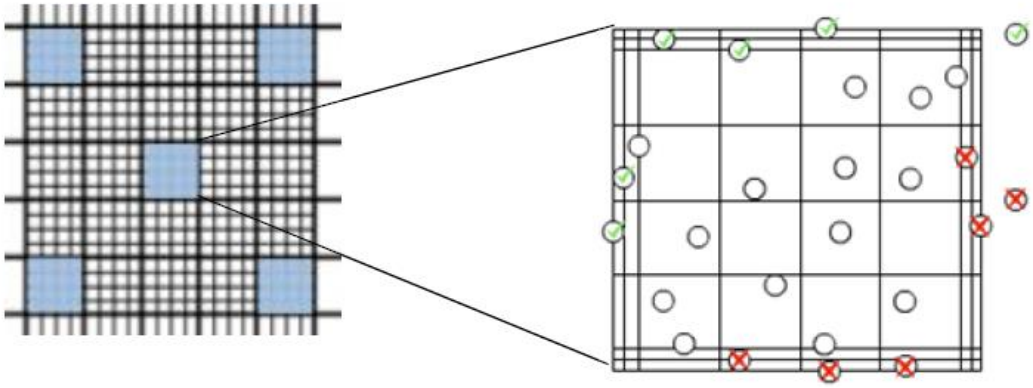

Figure 6-2. Haemocytometer counting system [361].

Record the number of live, dead leukocytes and squamous cells. Calculate the viable cell count and total cell concentration (cells/g sputum) as follows:

1. The total leukocyte count is calculated as the number of live + dead leucocytes.
2. To calculate the number of leucocytes per field:

   Total leukocytes ÷ number of fields used

3. Calculate the number if viable cells (%):

   Live cells ÷ total leukocytes x 100

4. Calculate the squamous cell contamination (%):

   Squamous cells ÷ (total leukocytes + squamous cells) x 100

5. To calculate the cell concentration:

   Leukocytes per field x dilution factor (2) x 10^4

   The volume of 1 large corner square is $1mm^2$ x 0.1mm, therefore a conversion factor of 10^4 is used: 0.1cm x 0.1cm x 0.01cm = 10^{-4} cm^3 or 10^{-4} ml. An adjustment is also made for the trypan blue dilution.

6. To calculate the total cell number:

   Cell concentration x S2 sample weight

7. To calculate the total cells/g sputum:

   Total cell number ÷ S1 sample weight (original sample weight)

6.7.9 Differential cell counts

- Next, Centrifuge the DTT cell suspension in S2 at 600g for 10 minutes at 4°C
- Split the resulting supernatant between four cryovials labelled *'DTT Supernatant'* and store at -80°C.
- Re suspend the cell pellet in an appropriate volume of d-PBS to give a concentration of $5x10^5$ cells/ml (calculated as: Total cell number ÷ $5x10^5$).
- Prepare and label two poly-lysine cytoslides with subject number, date and sample type. Pipette 75µl into one slide and 150µl to the other.
- Spin the cytoslides in a Shandon cytospin (Thermo Scientific) at 450 revolutions per minute (RPM) for 6 minutes.
- Allow the slides to air-dry and fix in methanol. Stain the 2 slides with RappiDiff II solution B and solution C.
- Trained research scientists in the Nottingham respiratory biomedical research unit then performed the differential cell counts.

6.8 Statistical analysis

All statistical analysis was performed using STATA 15 statistical software (Texas, USA). The participant baseline characteristics, lung function, hospital microbiology, concurrent medications and antibiotic data were analysed using paired t-tests, Chi-squared or Fisher's exact tests and Mann-Whitney U tests. Sputum neutrophil counts and quantitative microbiology were logarithmically transformed. Comparisons of urinary biomarkers and pro-inflammatory cytokines with quantitative microbiology, lung function (FEV_1), sputum and blood neutrophil concentration were performed using spearman's rank correlations, paired t-tests and Mann-Whitney U tests.

Time to next exacerbation and death (as dependent variables) were analysed using multivariable Cox proportional hazards models, adjusted for age, gender and lung function (FEV_1/L). Data were censored to the end of the study period on 30/11/2020. Proportional hazard assumptions were confirmed based on Schoenfeld residuals.

6.9 Summary

This chapter has described the methodology of delivering a prospective observational cohort study in non-CF bronchiectasis requiring IV antibiotic treatment for a pulmonary exacerbation. The clinical and microbiological factors along with quantitative PCR analysis are explored in Chapter 7, the urinary inflammatory biomarkers are described in Chapter 8, and the pro-inflammatory cytokines are described in Chapter 9.

Chapter 7. Exploring clinical and microbiological factors: Airway Infection in Bronchiectasis; a feasibility study.

7.1 Introduction

Bronchiectasis is a heterogeneous disease that is characterised by permanent bronchial dilatation, scarring and destruction of lung tissue, and recurrent respiratory infections. An initial infectious episode that then triggers an inflammatory response with subsequent bronchial wall damage encompasses the pathophysiological theory of “Cole’s vicious cycle”[362]. However, despite both acute and chronic pulmonary infections encompassing the natural history of bronchiectasis, the impact of them in combination substantially negatively impacts on the patients’ quality of life [363], disease progression and mortality [6]. Bronchiectasis is relatively common, with an estimated 212,000 individuals having the disease in the UK [3].

Frequent and recurrent exacerbations are associated with and increased airway and systemic inflammation [79], resulting in progressive lung damage [6]. As a result, it is a research and clinical priority to prevent or reduce exacerbations and their frequency. The early diagnosis and treatment of exacerbations are fundamental in the management of bronchiectasis, both in the short-term and also in terms of preserving lung tissue.

The aetiology of bronchiectasis exacerbations is generally assumed to be infectious in nature. Rosales *et al* identified *P. aeruginosa*, respiratory viruses, *Staphylococcus aureus*, *Streptococcus pneumonia*, *Haemophilus influenzae* and *Moraxella catharrhalis* as the most common microbiological causes of exacerbations [364]. However, the role of polymicrobial infections or interactions, viral and fungal infections are unclear.

This chapter explains the clinical results of a bronchiectasis observational cohort study of which the methodology is described in Chapter 6.

Due to the COVID-19 pandemic, the non-CF bronchiectasis feasibility study for quorum sensing molecules was delayed by the time of writing this thesis. As such, further analysis was performed to determine clinical, microbiological and inflammatory factors that may influence or reflect clinical response in pulmonary exacerbations, disease severity, time between exacerbations and mortality.

The aims addressed in this chapter were to determine:

- Baseline demographics of the participants including severity scores and bacterial colonisation status
- Intravenous antibiotic therapy (mean number of days, most frequently used and complications)
- Blood inflammatory markers at pulmonary exacerbation, following intravenous antibiotics and at clinical stability
- Spirometry results at pulmonary exacerbation, following intravenous antibiotics and at clinical stability
- Quality of life questionnaires: at exacerbation, after antibiotics and clinical stability
- The differential and quantitative white cell count, total bacterial load and *P. aeruginosa* load (culture and culture-independent) on sputum at pulmonary exacerbation, following intravenous antibiotics and at clinical stability
- Hospital microbiological culture and viral throat swab results at exacerbation, following intravenous antibiotics and at clinical stability
- Time to next pulmonary exacerbation and mortality

7.2 Participant recruitment and samples obtained

A total of 50 participants who were experiencing a pulmonary exacerbation that warranted IV antibiotics for non-CF bronchiectasis were recruited into the observational cohort study. On initial screening of participants for recruitment (defined in Chapter 6), no patients refused to take part in the study and all screened patients were eligible. Ten participants (20%) were treated in hospital for the entirety of their course of antibiotics, 28 (56%) were initially treated in hospital and subsequently completed their IV antibiotic course through the outpatient parenteral antibiotic team (OPAT) and 12 participants (24%) were recruited directly through the OPAT team as an admission avoidance. There were no patients lost to follow up, however 7 (14%) participants died before completion of the 3 visits; 2 died between visit 1 (within 72 hours of commencing IV antibiotics) and visit 2 (within 72 hours of completing IV antibiotics), and 5 died between visit 2 and 3 (clinical stability).

The median duration between commencement of IV antibiotics and recruitment of the participant was 0.5 days (IQR: 0 to 2), all participants were seen within 3 days of IV antibiotics except one participant who was seen after 3 days. The median duration between completion of IV antibiotics and visit 2 was 1 day (IQR: 0 to 1.5). All participants were seen within 3 days of stopping IV antibiotics except 2 participants who were unable to attend the review within 3 days (seen at 5 days and at 4 days). In addition, one patient was converted to 3 weeks of oral Ciprofloxacin after 2 days of IV antibiotics and was subsequently seen within 72 hours of completion of the oral antibiotic course. After completing IV antibiotics, the median duration to visit 3 at clinical stability was 6.1 weeks (IQR: 4.9 to 11.1).

For visit 1, 48 sputum samples, 50 plasma samples and 47 urine samples were collected from 50 participants. At visit 1, all samples were obtained at the same day of the visit except 3 sputum samples and 1 urine sample which were obtained 24 hours after initial recruitment. At visit 2, 38 sputum samples, 47 plasma samples and 45 urine samples were collected from 48 participants. In addition, all samples were

obtained on the same day of the visit 2. At visit 3, 34 sputum samples, 43 plasma samples and 39 urine samples were collected from 43 participants and were all obtained on the same day as the visit. In total, 120 sputum, 140 plasma and 131 urine samples were collected across the participant cohort.

7.3 Baseline demographics

The baseline demographics of the study population are summarised in Table 7-1. The median age of the participants was 71.2 years (age range: 43.7 to 92.9). Thirty participants were female (60%) and 20 were male (40%). Forty-six participants (92%) had an ethnicity of white Caucasian, whilst the remaining participants were Afro-Caribbean (4%) and Asian (4%). The majority of participants were ex-smokers (52%), whilst 34% had never smoked, 10% were current smokers and 4% had passive smoke exposure. The mean FEV_1 % predicted at clinical stability was 45% (SD±22.1). The median number of pulmonary exacerbations in the last year was 3, ranging from 0 to 12 (Figure 7-1). In addition, 46 participants (92%) had a severe bronchiectasis severity index, with the consequence that their 1 year estimated mortality rate ranged from of 8% to 10% and a hospitalisation rate over the same period from 17% to 53% [6].

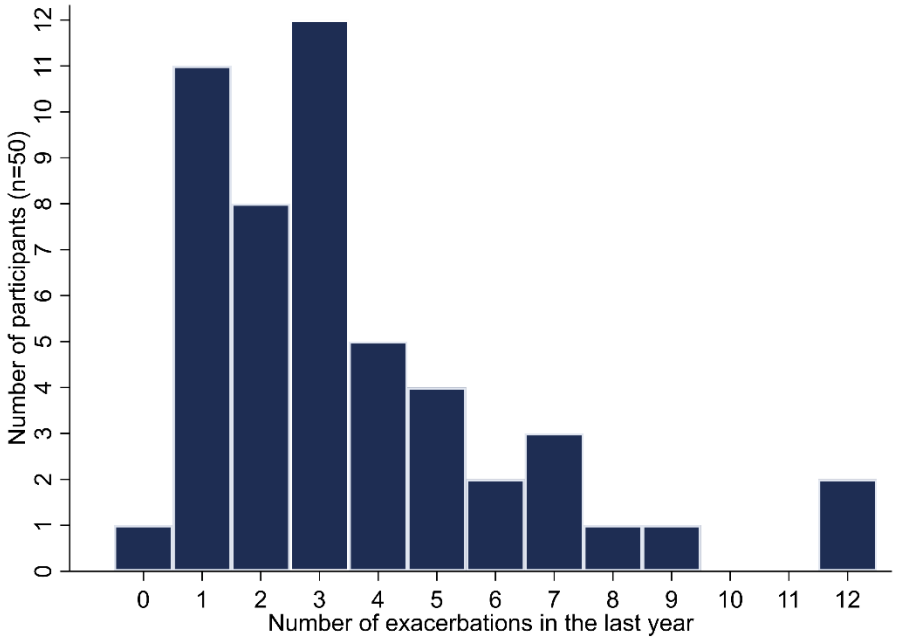

Figure 7-1. The Number of exacerbations in the preceding 12 months in non-CF bronchiectasis cohort, n=50.

Chronic colonisation was defined by the isolation of potentially pathogenic bacteria in sputum culture on two or more occasions, at least 3 months apart in a 1-year period [6, 365].

Thirty-two participants (64%) were identified as being chronically colonised by a micro-organism. Of those who were chronically colonised, 27 participants (84.4%) were colonised with *Pseudomonas aeruginosa* (non-mucoid 46.9%, mucoid 37.5%), 4 participants were colonised with *Haemophilus influenzae* (12.5%) and one patient was colonised with *Aspergillus fumigatus* (3.1%). The median duration of participants chronically colonised with any *P. aeruginosa* (n=27) was 5 years (IQR: 1.1 to 10.2). The median duration for non-mucoid colonisation was 3.6 years (IQR: 0.9 to 10.2) and mucoid was 6.9 years (IQR: 1.8 to 10.5). Nine participants (18%) had never isolated *P. aeruginosa* and 32 (64%) participants isolated *P. aeruginosa* within the last 12 months prior to recruitment. Five participants (10%) isolated new *P. aeruginosa* within the preceding 4 weeks prior to recruitment. The baseline demographics are summarised in Table 7-1.

The median age at diagnosis of the participants was known for 34 people and was 63.2 years old (range: 39.5 to 86.6). Half of the cohort (50%) had an unknown or idiopathic cause of bronchiectasis, whilst 32% had a post-infective aetiology. The main post-infective cause was pneumonia at 31.3%. Twenty-two participants (44%) had COPD as a co-morbidity and 13 participants (26%) had a diagnosis of asthma. In addition, the majority of participants (48%) had a Bronchiectasis Aetiology and Co-morbidity index of intermediate risk; which has an estimated 5 year mortality risk of 11.7% and a 5 year hospitalisation risk for severe exacerbation of 14.8% [366]. The aetiology and co-morbidities are summarised in Table 7-2. These results are comparable with a previous large European cohort study investigating aetiologies and baseline demographics within differing levels of bronchiectasis severity [27].

7.3.1 Antibiotic treatment

The median IV antibiotic duration was 13 days (IQR: 11 to 14). Eight participants (16%) had received an intravenous antibiotic within the last 4 weeks prior to pulmonary exacerbation and 32 participants (64%) had received an oral antibiotic within the previous 4 weeks (Table 7-3). A total of 25 participants had their intravenous antibiotic changed to another antibiotic or stopped early due to a variety of reasons such as outlined in Table 7-3 such as:

- referral to OPAT (therefore changing to a once a day regimen),
- clinical decision, inadequate response to therapy
- rash
- *C. difficile* infection
- self-discharge
- acute kidney injury
- death

The main IV antibiotic that the participants completed their IV antibiotic course on was Ceftazidime (30%). This was closely followed by Piperacillin/Tazobactam (24%). A total of 8 participants (16%) received combination antibiotics for known previous multi-drug resistant *P. aeruginosa* infections. The antibiotic treatment is summarised in Table 7-3.

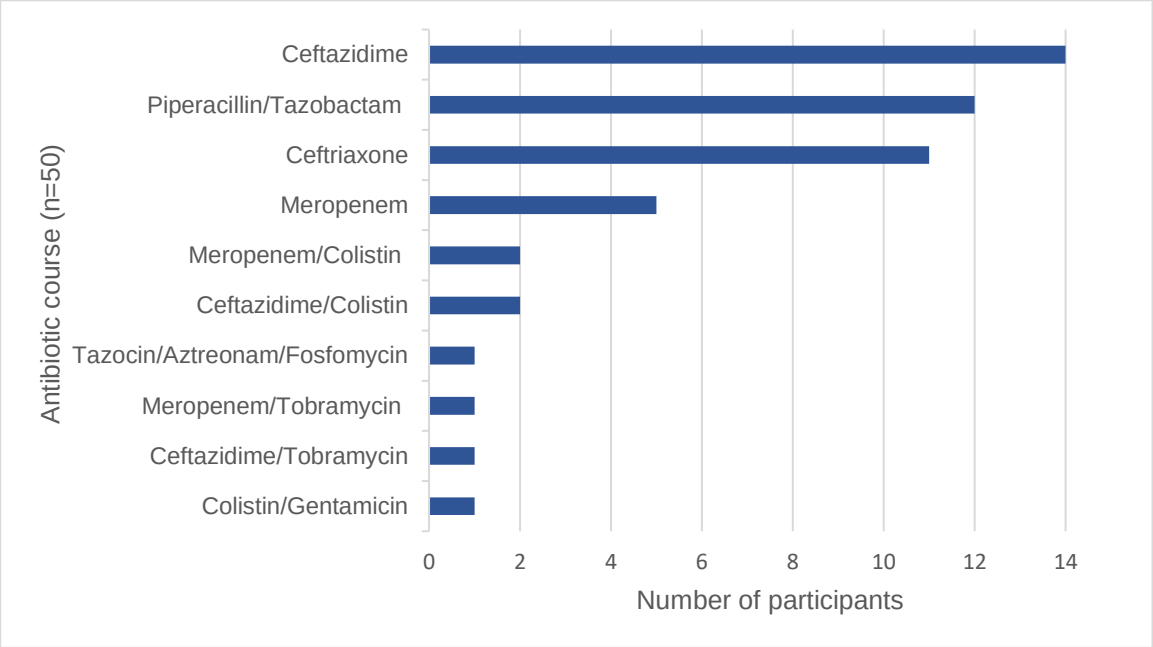

Figure 7-2. The antibiotic course completion combinations that were administered to the 50 participants for an infective exacerbation of bronchiectasis.

Table 7-1. Baseline demographics of the non-CF bronchiectasis cohort.

Variable (n=50)	Baseline
Age in years: median (range)	71.2 (43.7 to 92.9)
Gender: n (%)	
- Male	20 (40)
- Female	30 (60)
Ethnicity: n (%)	
- White	46 (92)
- Afro-Caribbean	2 (4)
- Asian	2 (4)
Weight: mean (SD)	66.6 (± 20.8)
BMI: mean (SD)	23.4 (± 6.6)
Smoking status: n (%)	
- Never	17 (34)
- Ex-smoker>12 months	26 (52)
- Current	5 (10)
- Passive exposure	2 (4)
FEV_1% predicted: mean (SD)	45 (± 22.1)
Hospital admissions/ 2 yrs: median (range)	3 (0 to 21)
Exacerbations/yr: median (range)	3 (0 to 12)
Bronchiectasis Severity Index: n (%)¥	
- Mild (0-4)	2 (4)
- Moderate (5-8)	2 (4)
- Severe (9+)	46 (92)
Never isolated *P. aeruginosa*	9 (18)
P. aeruginosa in last 12 months*	32 (64)
New isolation of *P. aeruginosa* in last 4 weeks	5 (10)
Chronic colonisation	
- non-mucoid *P. aeruginosa*	15 (46.9)
- mucoid *P. aeruginosa*	12 (37.5)
- *Haemophilus influenzae*	4 (12.5)
- *Aspergillus fumigatus*	1 (3.1)
Years colonised with *P. aeruginosa:* median (IQR)	5 (1.1 to 10.2)
Maintenance therapy: n (%)	
- Azithromycin	19 (38)
- Colomycin nebulisers	6 (12)
- Inhaled steroids	31 (62)

¥ 0-4 Mild Bronchiectasis: 1 year outcomes: 0 - 2.8 % mortality rate, 0 - 3.4 % hospitalisation rate, 4 year outcomes: 0 - 5.3 % mortality rate, 0 - 9.2 % hospitalisation rate, 5 – 8 Moderate Bronchiectasis:1 year outcomes: 0.8 - 4.8 % mortality rate, 1.0 - 7.2 % hospitalisation rate, 4 year outcomes: 4 % - 11.3 % mortality rate, 9.9 - 19.4 % hospitalisation rate, 9 +Severe Bronchiectasis:
1 year outcomes: 7.6 % - 10.5 % mortality rate, 16.7 - 52.6 % hospitalisation rate
4 year outcomes: 9.9 - 29.2 % mortality, 41.2 - 80.4 % hospitalisation rate
*; n=6 patients had multi drug resistant *P. aeruginosa* in the last 12 months (resistance to at least 1 agent from 3 antibiotic classes)

Table 7-2. Aetiology and co-morbidities of the non-CF bronchiectasis cohort.

Variable	Baseline
Age at diagnosis: median (range)§	63.2 (39.5 to 86.6)
Aetiology: n (%)	
- Unknown/idiopathic	25 (50)
- Post-infective: n (%)	16 (32)
• Pneumonia	5 (31.3)
• Whooping cough	4 (25)
• Measles + Pneumonia	2 (12.5)
• Tuberculosis	2 (12.5)
• Whooping cough + Pneumonia*	1 (6.3)
• Whooping cough + Pneumonia + Tuberculosis*	1 (6.3)
• Whooping cough + Measles + Pneumonia*	1 (6.3)
- Rheumatoid arthritis	4 (8)
- ABPA	2 (4)
- GORD	1 (2)
- IBD	1 (2)
- Immune deficiency	1 (2)
Co-morbidity: n (%)	
- COPD	22 (44)
- Asthma	13 (26)
- IHD	8 (16)
- Diabetes	5 (10)
- Pulmonary hypertension	4 (8)
- Haematological malignancy	2 (4)
- Inflammatory bowel disease	2 (4)
- Chronic liver disease	1 (2)
- Iron deficiency anaemia	1 (2)
BACI: n (%)±	
- Low risk (0)	11 (22)
- Intermediate risk (1-5)	24 (48)
- High risk (>6)	15 (30)

§; number of participants with exact date of diagnosis known: n=34, *; participants had multiple previous infections and therefore exact aetiology of post-infectious cause is not known,

± Bronchiectasis aetiology co-morbidity index; 0: Low Risk: Estimated 5 year mortality risk 3.5%, Estimated risk of hospitalisation for severe exacerbation 11.7% over 5 years, 1-5: Intermediate Risk: Estimated 5 year mortality risk 11.7%, Estimated risk of hospitalisation for severe exacerbation 14.8% over 5 years, 6 or more: High Risk: Estimated 5 year mortality risk 34.9%, Estimated risk of hospitalisation for severe exacerbation 36% over 5 years.

Table 7-3. Antibiotic treatment for pulmonary exacerbation.

Variable	Participants
IV antibiotic in the last 4 weeks±	8 (16)
Oral antibiotic in the last 4 weeks€	32 (64)
Total IV antibiotic days: median (IQR)	13 (11 to 14)
IV antibiotic treatment for exacerbation: n (%)*	
Single agent therapy:	42 (84)
Ceftazidime	15 (30)
Piperacillin/Tazobactam	12 (24)
Ceftriaxone	10 (20)
Meropenem	5 (10)
Dual agent therapy:	8 (16)
Meropenem+Colistin	2 (4)
Ceftazidime+Colistin	2 (4)
Colistin+Gentamicin	1 (2)
Ceftazidime+Tobramycin	1 (2)
Meropenem+Tobramycin	1 (2)
Piperacillin/Tazobactam+Aztreonam+Fosfomycin	1 (2)
Antibiotic changed: n(%)	
OPAT	8 (32)
Clinician decision	6 (24)
Death	3 (12)
C. difficile infection	2 (8)
Not responding	2 (8)
Rash	2 (8)
Self-discharge	1 (4)
AKI	1 (4)

* The IV antibiotic that the course was completed on; OPAT: Outpatient Parenteral antibiotic therapy, AKI: Acute kidney injury.
± Piperacillin/Tazobactam n=4, Co-amoxiclav n=4, Meropenem n=2, Gentamicin n=1. Ceftazidime n=1. Some participants had more than one IV antibiotic in the last 4 weeks prior to recruitment.
€ Doxycycline n=13, Ciprofloxacin n=9, Co-amoxiclav n=6, Levofloxacin n=3, Clarithromycin n=3, Cefalexin n=3, Amoxicillin n=3, Co-trimoxazole n=2. Some participants had more than one oral antibiotic in the last 4 weeks prior to recruitment.

7.3.2 Hospital microbiology (qualitative) results

The hospital microbiology results are summarised in Table 7-4. At the beginning of a pulmonary exacerbation, 47 (94%) participants produced a sputum sample for hospital microbiological culture. Within 7 days of a pulmonary exacerbation, 18 (38.3%) participants out of 47 isolated *P. aeruginosa*; 11 participants isolated non-mucoid (23.4%) and 7 isolated mucoid strains (14.9%). However, 25 participants (50%) isolated *P. aeruginosa* from sputum within 4 weeks of pulmonary exacerbation. Three participants isolated *Haemophilus influenzae* (6.4%), and one participant isolated Staphylococcus aureus (2.13%) from sputum. *Haemophilus influenzae* was isolated with the absence of other pathogens but Staphylococcus aureus was co-isolated with mucoid *P. aeruginosa* in the same participant.

All participants except the first participant (AIB001) also had a viral throat swab taken at pulmonary exacerbation. Respiratory viruses were isolated in 10 participants and 1 participant isolated more than one virus (Rhinovirus/Enterovirus and Adenovirus). Multi-drug resistant (MDR) *P. aeruginosa* was defined as resistance to at least one antibiotic from 3 separate antimicrobial classes. The classes included were predominantly: aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems and fluoroquinolones [367]. MDR was found in 2 participants both at pulmonary exacerbation and after IV antibiotics and 3 participants at clinical stability. All participants with MDR had previously isolated MDR *P. aeruginosa* in the preceding 12 months.

After IV antibiotics, 21 out of 48 (43.8%) participants produced a sputum sample for hospital microbiological culture. Compared to the beginning of pulmonary exacerbation, the isolation of *P. aeruginosa* in sputum reduced to 4 out of 21 participants (19%, p=0.033); 3 with non-mucoid and 1 with a mucoid strain. At clinical stability, 26 out of 43 (60.5%) participants produced sputum samples for hospital microbiological culture. Compared to the beginning of a pulmonary exacerbation, *P. aeruginosa* was isolated from with 15 participants (57.7%, p=0.013); at clinical stability; 8 with non- mucoid (30.8%) and 7 with a mucoid strain (26.9%). Viral throat

swabs were not routinely taken after IV antibiotics or at clinical stability unless symptomatic of a viral infection. One patient isolated both influenza A and Rhinovirus/Enterovirus after IV antibiotics for a pulmonary exacerbation.

Table 7-4. Microbiology hospital results for non-CF bronchiectasis cohort.

Organism: n (%)	At exacerbation	Post exacerbation	Clinical stability§
	N=47	N=21	N=26
Any *P. aeruginosa*	18 (38.3)	4 (19.05)	15 (57.7)
Fisher's exact *p*-value		**0.033ª**	**0.013¥**
non-mucoid *P. aeruginosa**	11 (23.4)*	3 (14.3)*	8 (30.8)
mucoid *P. aeruginosa**	7 (14.9)*	1 (4.8)*	7 (26.9)
Multi drug resistant *P. aeruginosa*§	2 (4.3)	2 (9.5)	3 (11.5)
Haemophilus influenzae	3 (6.3)	0	1 (3.8)
Staphylococcus aureus	1 (2.12)	0	0
Stenotrophomonas maltophilia	0	0	1 (3.8)
Respiratory commensals	25 (53.2)	17 (81)	10 (38.5)
Viruses: n (%)			
Enterovirus/Rhinovirus°	8 (16)	1 (2)	0
Respiratory syncytial virus	1 (2)	0	0
Adenovirus	1 (2)	0	0
Bocavirus	1 (2)	0	0
Influenza A	0	1 (2)	0

§: Multi drug resistance is defined as resistant to at least 1 antibiotic in 3 or more classes
*; defined as positive within 7 days to visit date. §; microbiology results at clinical stability are defined as all the positive microbiology since post-exacerbation visit, Only respiratory commensals isolated in the absence of a pathogen were included. °The hospital laboratory is unable to differentiate between Enterovirus or Rhinovirus on viral throat swab
At exacerbation sputum sent to hospital microbiology N=47 (1 sputum had no growth), Post exacerbation sputum sent to hospital microbiology N=21, Clinical stability sputum sent to hospital microbiology N=26, ª; Fisher's exact p-value between beginning and end of pulmonary exacerbation, ¥; Fisher's exact p-value between beginning of a pulmonary exacerbation and clinical stability, p-values in bold are <0.05.

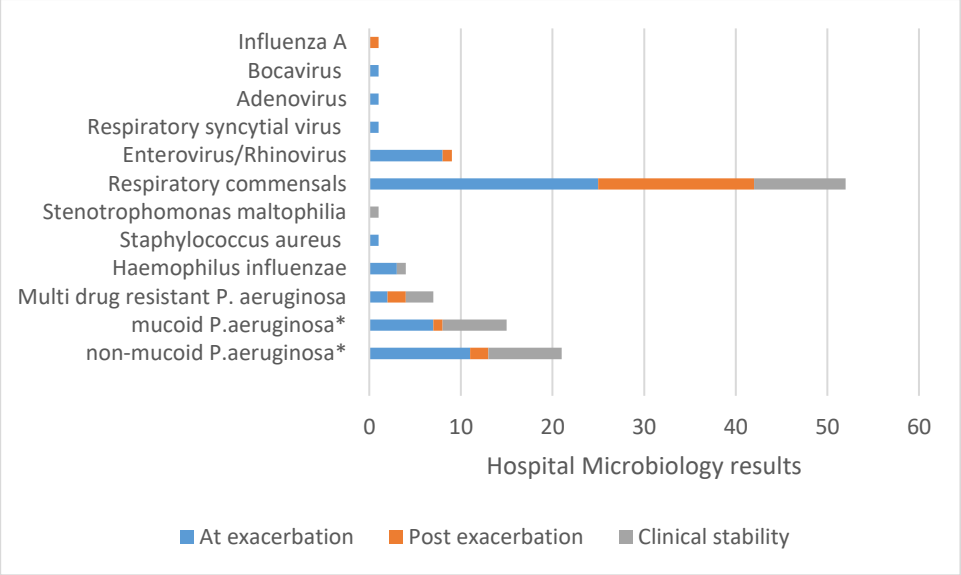

Figure 7-3. Positive microbiology hospital results at the beginning of IV antibiotics, the end of IV antibiotics and clinical stability in non-CF bronchiectasis cohort.

7.3.3 Summary of baseline demographics

The aetiologies of bronchiectasis in our cohort were consistent with those observed in previous studies in that the majority of patients had an idiopathic or unknown cause, ranging from 26% to 74% [26].

A large European cohort study that prospectively enrolled 1,258 patients from 7 international and national sites showed some similar baseline characteristics to our data [27]. The median age of participants was 67 (58 to 75) and the BMI was 25 (21 to 28). The aetiologies of bronchiectasis showed an idiopathic cause in 40%, post-infective cause in 20%, IBD 1.9%, ABPA in 4.5%, Asthma in 3.3% and Immunodefiency in 5.8%.

However, compared to the European cohort, our participants were more likely to be smokers (62% vs 36%), colonised with *P. aeruginosa* (54 % vs 15%) and had higher BSI scores (92% had a severe BSI score vs 29%).

Furthermore, one of the largest UK based registries (BronchUK) recruited 1403 patients from 13 secondary care sites [368]. In that study, there were some similarities with our cohort; 62% were female and 38% were male, the median age was 67 (61 to 73), patients were predominately Caucasian at 93 % and the BMI was 26.5 (22.3–29.3). Common aetiologies were idiopathic (40%) and post infectious (34%). COPD and Asthma were either common comorbidities or suspected aetiologies (16–21% and 3–39%) respectively.

However, there were some notable differences with our data. The mean FEV_1 % predicted in our data set was 45% compared to a median FEV1 % predicted of 77% in the BronchUK dataset. In addition, compared to 52% ex-smokers and 10% current smokers in our cohort, the majority of patients in the BronchUK dataset were never smokers at 59%, 37% were ex-smokers and 4% were current smokers. In our cohort, 44% of participants had COPD as a co-morbidity. In addition, only 39% of patients in the BronchUK dataset had 3 exacerbations or more in the prior 12 months, compared to our cohort where the median number of exacerbations in the preceding 12 months was 3.

In contrary to our data, *Haemophilus influenzae* was the commonest organism isolated at 19%, and only 27% isolated *P. aeruginosa* in the preceding 2 years, compared to our data where 64% had isolated *P. aeruginosa* in the last year. By contrast, the Bronchiectasis severity index (BSI) was; mild= 29%, moderate= 48%, severe 23%.

In summary, the baseline demographics such as aetiology, age and BMI were similar in our cohort compared to two large international and national cohort studies. However, in comparison, our cohort had a higher prevalence of COPD, exacerbation rate, severity scores, a lower lung function, and the majority had chronic colonisation with *P. aeruginosa.* This may be due to our study recruiting patients at the start of a pulmonary exacerbation necessitating hospitalisation, therefore selecting patients from the more severe end of the spectrum of disease.

7.4 Clinical and microbiological outcomes

7.4.1 Comparisons between the beginning and end of a pulmonary exacerbation

Using paired t-tests, there were no statistical significances between the lung function measurements at the beginning and end of IV antibiotics for pulmonary exacerbation. At the beginning of IV antibiotics, the mean FEV_1/L and FEV_1 % predicted was 1.09 (SD± 0.6) and 42.9% (SD± 21.5), respectively. After IV antibiotics for a pulmonary exacerbation, the FEV_1/L and FEV_1 % predicted increased to 1.21 (SD± 0.8, p=0.162) and 46.7% (SD± 25.5, p=0.138). Similarly, the mean peak flow increased from 183.8 (SD± 113.5) to 202.6 (SD± 135.6, p=0.144, Table 7-5).

The median serum CRP at exacerbation was 13.5 (IQR: 2 to 24) and this decreased significantly to 5 (IQR: 0 to 12) after exacerbation, p=0.013, Table 7-5. Similarly, At the beginning of exacerbation, the median total blood white cell count and blood neutrophil count was 9.2 (IQR: 8 to 13.6) and 6.9 (IQR: 5.2 to 11.30) respectively, and decreased to 8.3 (IQR: 6.6 to 10.3), p=0.016, and 5.4 (IQR: 4.3 to 8.2), p=0.002, after exacerbation, respectively. The mean sputum neutrophils (log cells/g) decreased from 7.21 (SD± 0.6) to 6.72 (SD±0.63, p= 0.011, Table 7-5), after IV antibiotic treatment for a pulmonary exacerbation.

There was no difference on quantitative total bacterial load or *P. aeruginosa* load (log CFU/g) measured on culture between the beginning and end of IV antibiotics. However, there was evidence of bacterial clearance in sputum samples as 20 out of 47 participants (42.6%) isolated *P. aeruginosa* in sputum for quantitative microbiology at the beginning of exacerbation, compared to 12 out of 34 (35.3%) after antibiotics for an exacerbation (p=0.004, Table 7-5).

The total quantitative *P. aeruginosa* load measured by PCR showed no significant differences between the beginning of a pulmonary exacerbation and after treatment. However, by using a photoreactive dye, propidium monoazide (PMA), we were able to

differentiate between viable and compromised *P. aeruginosa* cells. Using this technique, there was a significant difference between the amount of live cells between the beginning and end of a pulmonary exacerbation; 3.08 (SD± 2.61) log_{10} CFU/g to 1.90 (SD ± 2.69) log_{10} CFU/g (p=0.011, Table 7-5).

The strongest difference between the beginning and end of pulmonary exacerbation was measured by the median quality of life questionnaire score which improved from 31.25 (IQR; 22.0 to 45.8) to 63 (IQR; 48.1 to 70.4, p<0.001).

7.4.2 Comparisons between the beginning of a pulmonary exacerbation and clinical stability

Using paired t-tests, there were no statistical significances between the lung function measurements at the beginning of a pulmonary exacerbation and clinical stability. At the beginning of IV antibiotics, the mean FEV_1/L and FEV_1 % predicted was 1.16 (SD ± 0.6) and 44.8% (SD± 21.1), respectively. At clinical stability, the FEV_1/L and FEV_1 % predicted increased to 1.23 (SD± 0.7, p=0.334) and 47.2% (SD± 25.5, p=0.267). The mean peak flow increased from 196.8 (SD± 113.9) to 208.4 (SD± 114.6) between pulmonary exacerbation and clinical stability (p=0.246, Table 7-5).

There was no difference between sputum neutrophils (log cells/g) from pulmonary exacerbation and clinical stability, however, the mean total bacterial load and *P. aeruginosa* load (log CFU/g) measured by culture increased from 2.28 (SD± 3.0) to 3.70 (SD± 3.4, p=0.045), and 6.53 (SD± 1.05) to 7.66 (SD± 0.7, p<0.001), respectively. In addition, out of 32 sputum samples provided for quantitative microbiology a higher proportion were positive for *P. aeruginosa* at clinical stability; 19 (59.4%) isolated *P. aeruginosa* compared to 42.6% at the beginning of pulmonary exacerbation (p=0.026, Table 7-5). There were no significant differences on the total or viable cells measured by quantitative *P. aeruginosa* PCR between pulmonary exacerbation and clinical stability.

Again, the strongest difference was measured by the median quality of life questionnaire score which improved from 31.5 (IQR; 22.0 to 45.8) at pulmonary exacerbation to 59.3 at clinical stability (IQR; 44.4 to 70.4, $p<0.001$).

Table 7-5. Comparison of clinical and quantitative microbiological outcomes between the beginning of a pulmonary exacerbation, the end of a pulmonary exacerbation and clinical stability.

Variable	At exacerbation	After exacerbation	*p*-value
Mean Absolute FEV_1 in L	1.09 (± 0.60)	1.21 (±0.82)	0.162
Mean FEV_1 % predicted)	42.94 (±21.50)	46.71 (± 25.49)	0.138
Mean Peak Flow L/min	183.82 (±113.45)	202.59 (± 135.60)	0.144
Median Serum CRP	13.50 (2.00;24.00)	5.00 (0;12.00)	**0.013**
Median Blood total WCC¥	9.20 (8.00;13.60)	8.30 (6.62;10.34)	**0.016**
Median Blood neutrophil count¥	6.90 (5.18;11.30)	5.40 (4.30;8.20)	**0.002**
Mean Sputum neutrophils cell/g*	7.21 (±0.58)	6.72 (±0.63)	**0.011**
Mean PsA load CFU/g*	2.73 (± 3.04)	2.47 (± 3.46)	0.652
No. of PsA positive sputum	20 (42.55)	12 (35.29)	**0.004**
Mean Total bacterial load CFU/g*	6.54 (± 0.97)	6.69 (± 1.14)	0.523
Mean Total PsA PCR (live+dead)*	4.66 (±3.25)	4.14 (± 3.03)	0.331
Mean Live PsA PCR*	3.08 (± 2.61)	1.90 (± 2.69)	**0.011**
Median QOL Questionnaire	31.45 (22.00;45.80)	63.00 (48.10;70.40)	**<0.001**
	At exacerbation	Clinical Stability	*p*-value
Mean Absolute FEV_1 in L	1.16 (± 0.59)	1.23 (±0.74)	0.334
Mean FEV_1 % predicted)	44.83 (±21.10)	47.22 (± 22.71)	0.267
Peak Flow L/min: mean (SD)	196.79 (±113.99)	208.40 (± 114.60)	0.246
Mean Sputum neutrophils cell/g*	7.13 (±0.62)	7.09 (±0.58)	0.723
Mean PsA load CFU/g*	2.29 (± 3.04)	3.71 (± 3.41)	**0.045**
No. of PsA positive sputum	20 (42.55)	19 (59.38)	**0.026**
Mean Total bacterial load CFU/g*	6.53 (± 1.06)	7.66 (± 0.66)	**<0.001**
Mean Total PsA PCR (live+dead)*	4.19 (± 3.35)	5.21 (± 3.05)	0.058
Mean Live PsA PCR*	2.66 (± 2.71)	3.14 (± 3.01)	0.201
Median QOL Questionnaire	31.45 (22.00;45.80)	59.30 (44.40;70.40)	**<0.001**

At exacerbation n=50, Post exacerbation n=48, Clinical stability n=43, FEV_1; Forced expiration value in 1 second, L; litre, Mean variables are expressed as standard deviation in brackets, median variables are expressed as interquartile range in brackets, WCC; white cell count, *; log^{10}, QOL; quality of life questionnaire. CFU; colony forming units, g; gram. ¥;10^9/L, PsA; *P. aeruginosa*. °The number of sputum samples that grew *P. aeruginosa* at exacerbation was 20 out of 47 samples (42.55%), The number of sputum samples that grew *P. aeruginosa* post exacerbation was 12 out of 34 samples (35.29%). The number of sputum samples that grew *P. aeruginosa* at clinical stability was 19 out of 32 samples (59.38%).
Data for Blood total WCC, neutrophil count and serum CRP not available for clinical stability.
If variables are described as mean (SD), p-values were derived from paired t-tests, if variables are described as median (IQR), p-values are derived from Wilcoxon matched pairs signed rank tests. °;For comparison of PsA numbers in sputum the Fisher's exact test was used.
p-values in bold are significant if <0.05.

7.4.3 Cross-sectional correlations of clinical and microbiological outcomes

7.4.3.1 Gender, age and BMI

In agreement with previous literature, we hypothesised that a higher age and a lower body mass index (BMI) would be associated with lower pulmonary function [369, 370]. The FEV_1/L measured at the beginning of IV antibiotics for a pulmonary exacerbation had a negative correlation with participant age (r=-0.281, *p*=0.048, Figure 7-4). There was no correlation with FEV_1/L and body mass index (r=0.221, *p*=0.124). However, the peak flow min/L at exacerbation was negatively correlated with age (r=0.290, *p*=0.041) and had a positive correlation with BMI (r=0.280, *p*=0.049, Figure 7-4).

It is acknowledged in literature that females with bronchiectasis have a reduced lung function compared to males and as such, we hypothesised lung function would be consistently lower in females compared to males [371]. At the start of a pulmonary exacerbation, mean FEV_1/L was lower in females; (0.89, SD±0.52) compared to males; (1.36 SD± 0.62, *p*=0.006). At end of a pulmonary exacerbation, mean FEV_1/L was again lower in females; (0.95, SD±0.46) compared to males; (1.61 SD± 1.07, *p*=0.006). In addition, at clinical stability mean FEV_1/L was lower in females; (0.99, SD±0.44) compared to males; (1.59 SD± 0.96, *p*=0.009, Figure 7-5).

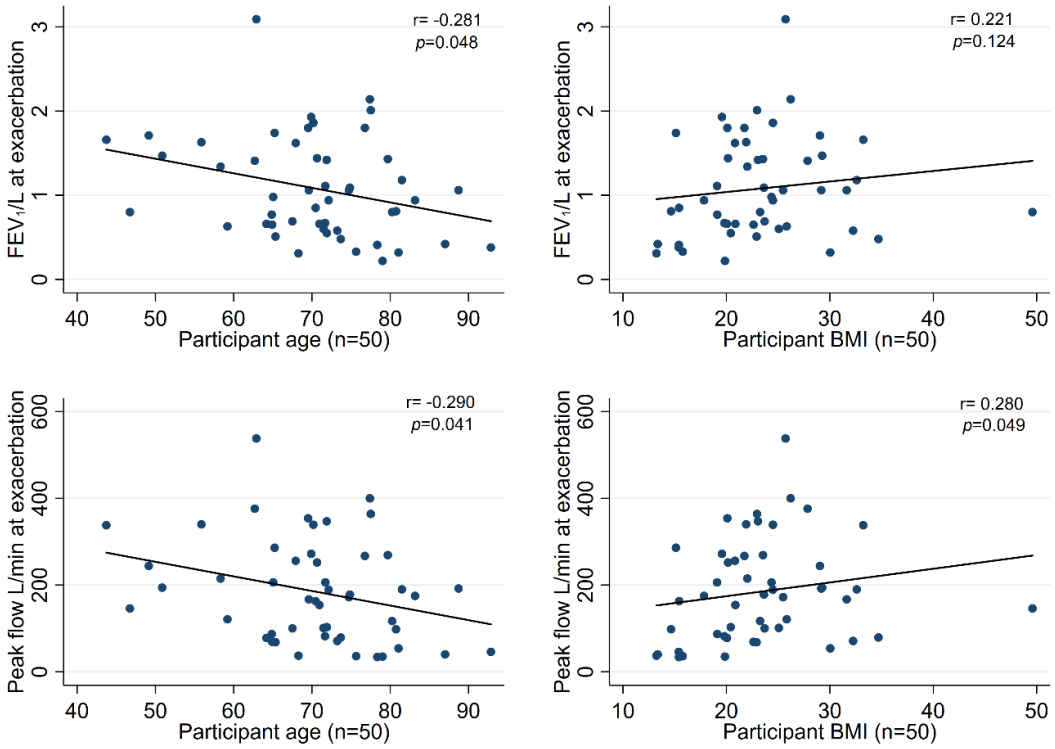

Figure 7-4. Correlations between FEV_1/L and peak flow L/min at pulmonary exacerbation with age and body mass index, r=Spearman's rho coefficient.

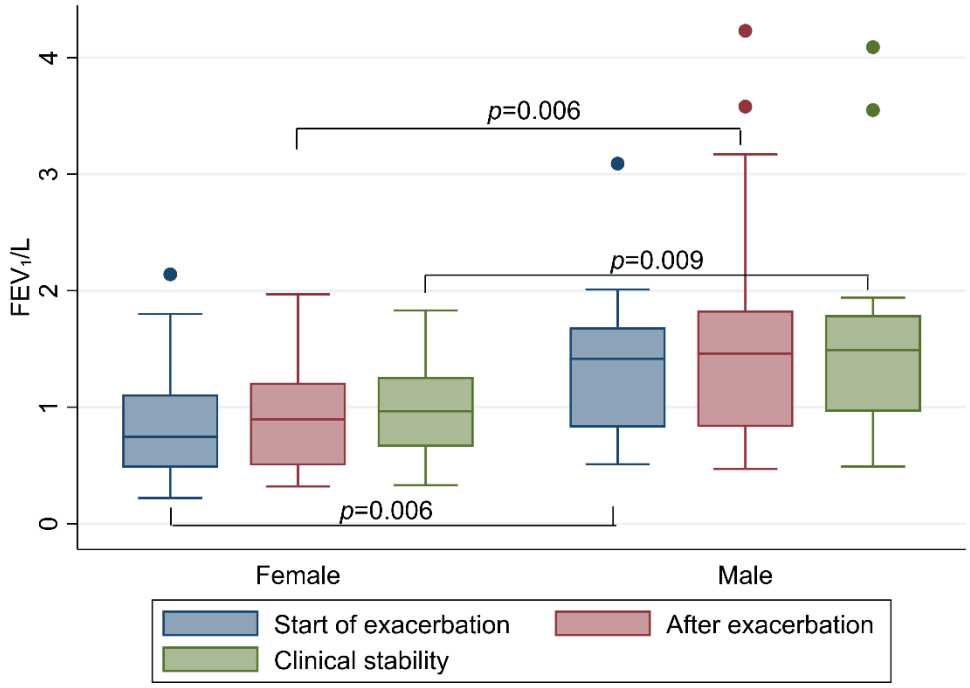

Figure 7-5. Gender differences in FEV_1/L.

7.4.3.2 Markers of inflammation and clinical parameters

At the beginning of IV antibiotics for pulmonary exacerbation, there were no correlations noted between lung function (FEV_1/L, FEV_1 % predicted or Peak flow L/min) and quality of life scores with sputum neutrophils, blood neutrophil count or serum CRP (Table 7-6). In addition, quality of life scores did not correlate with lung function parameters.

After treatment with IV antibiotics for a pulmonary exacerbation, the FEV_1/L correlated negatively with the sputum neutrophil count (r=-0.435 *p*=0.049). There were no correlations between lung function parameters and quality of life scores. The blood neutrophil count correlated negatively with FEV_1/L (r=0.382, *p*=0.009), FEV_1 % predicted (r=-0.424, *p*=0.004) and Peak flow L/min (r=-0.460, *p*=0.002).

At clinical stability, there were no correlations with lung function parameters and quality of life scores. However, the sputum neutrophil count correlated positively with the quality of life score (r=0.499, *p*=0.025).

Table 7-6. Correlations with clinical and inflammatory parameters at the beginning of IV antibiotics, the end of IV antibiotics and clinical stability.

	Spearman's Correlation Coefficient, r (*p*-value)		
At exacerbation	Sputum neuts¥	Blood neuts§	Serum CRP
FEV_1/L	0.234 (0.191)	-0.214 (0.136)	-0.112 (0.438)
FEV_1 % pred	-0.307 (0.082)	-0.161 (0.264)	-0.115 (0.427)
QOL	0.205 (0.254)	-0.074 (0.608)	-0.013 (0.927)
Peak flow L/min	-0.182 (0.311)	-0.157 (0.276)	-0.180 (0.211)
Post exacerbation			
FEV_1/L	**-0.435 (0.049)**	**-0.382 (0.009)**	-0.219 (0.154)
FEV_1 % pred	-0.423 (0.056)	**-0.424 (0.004)**	-0.216 (0.159)
QOL	-0.197 (0.379)	-0.267 (0.073)	-0.210 (0.167)
Peak flow L/min	-0.379 (0.090)	-**0.460 (0.002)**	-0.155 (0.316)
Clinical Stability			
FEV_1/L	-0.143 (0.548)		
FEV_1 % pred	-0.071 (0.767)		
QOL	**0.499 (0.025)**		
Peak flow L/min	-0.036 (0.880)		
BSI score	0.186 (0.433)		

FEV_1; Forced expiration value in 1 second, L; litre, QOL; quality of life questionnaire, ¥;log cells/g, *; log CFU/ml, §;10^9/L, Data for Blood total WCC, neutrophil count and serum CRP not available for clinical stability. BSI; Bronchiectasis severity index, , *p*-values in bold are significant if <0.05, r; Spearman's rho coefficient

7.4.3.3 Quantitative microbiology and clinical parameters

P. aeruginosa load measured by culture and PCR were consistently positively correlated at the beginning of a pulmonary exacerbation; r=0.764, *p*<0.001, after treatment; r=0.879, *p*<0.001, and at clinical stability; 0.728, *p*<0.001 (Figure 7-6).

However, at the beginning of a pulmonary exacerbation, 7 sputum samples were positive for *P. aeruginosa* measured by PCR but failed to grow by culture. In contrast only 1 sputum sample was detected by culture that was not detected by PCR. At the end of a pulmonary exacerbation, 2 sputum samples were positive for *P. aeruginosa* measured by PCR that was not detected in culture and 1 sputum sample was

detected by culture only. At clinical stability, 1 sample was detected by PCR only and 2 samples were detected only by culture in the absence of PCR.

This discrepancy may be explained by multiple reasons. Culture is dependent on adequate environmental conditions such as incubation temperatures and therefore may be prone to error and underestimation. In addition, as a result of anti-microbial therapy, the *P. aeruginosa* may have been present but at insufficient quantities to permit adequate growth by culture. Lastly, although PCR techniques are reliable and based on robust methodology, the live/dead cell separation technique used in this analysis remains a novel new way of enumerating bacterial cells and may lack standardisation and validation in this cohort.

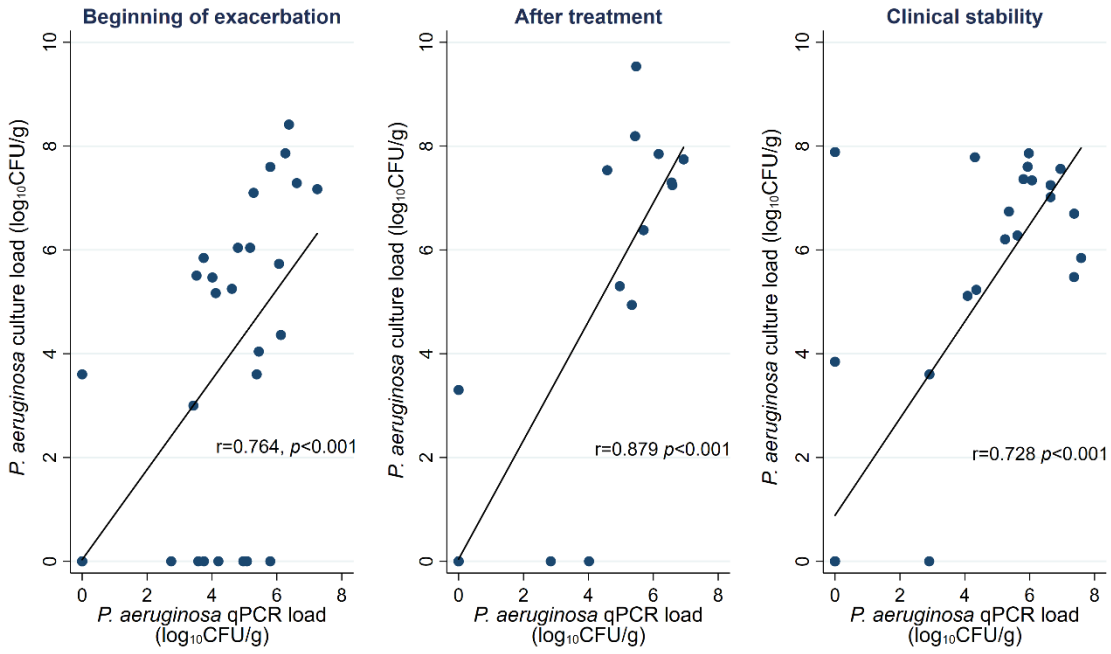

Figure 7-6. The correlation of *P. aeruginosa* quantitative load measured by culture and PCR.

At the beginning of a pulmonary exacerbation, there were no correlations for lung function, quality of life scores, sputum and blood neutrophils and serum CRP with either total bacterial load or *P. aeruginosa* load measured both by culture and PCR (Table 7-7).

After treatment with IV antibiotics for a pulmonary exacerbation, the FEV_1/L correlated negatively with *P. aeruginosa* load measured by culture; (r=-0.355, *p*=0.043), and *P. aeruginosa* load measured by PCR correlated negatively with FEV_1% predicted; (r= -0.398, *p*=0.018, Table 7-7). In addition, both *P. aeruginosa* load measured both by culture and PCR correlated positively with sputum neutrophils; (r=0.484, *p*=0.023 and r=0.563, *p*=0.006, respectively), and serum CRP; (r=0.368, *p*=0.038 and r=0.338, *p*=0.047, respectively). After treatment, total bacterial load also correlated positively with sputum neutrophil count; (r=0.568, *p*=0.011).

At clinical stability, total bacterial load measured on blood agar correlated negatively with FEV_1/L (r=-0.420, *p*=0.029) and peak flow L/min (r=-0.499, *p*=0.008) and correlated positively with the bronchiectasis severity score (r=0.455, *p*=0.017, Table 7-7). There were no correlations with any clinical measures at clinical stability with *P. aeruginosa* load measured by culture or PCR.

Table 7-7. Correlations with clinical and microbiological parameters at the beginning of IV antibiotics, the end of IV antibiotics and clinical stability.

	Spearman's Correlation Coefficient, r (p-value)		
Total Blood load	Exacerbation	Post Exacerbation	Clinical stability
FEV_1/L	0.072 (0.637)	-0.185 (0.327)	**-0.420 (0.029)**
FEV_1 % pred	0.163 (0.286)	-0.205 (0.277)	-0.365 (0.062)
Peak flow L/min	0.058 (0.707)	-0.093 (0.624)	**-0.499 (0.008)**
QOL	0.062 (0.684)	-0.231 (0.211)	0.006 (0.978)
Sputum neuts	-0.008 (0.965)	**0.568 (0.011)**	0.318 (0.248)
Blood neuts	-0.202 (0.184)	-0.124 (0.513)	
CRP	-0.037 (0.809)	0.158 (0.413)	
BSI score			**0.455 (0.017)**
PsA Culture Load	Exacerbation	Post Exacerbation	Clinical stability
FEV_1/L	-0.036 (0.808)	**-0.355 (0.043)**	-0.210 (0.249)
FEV_1 % pred	0.033 (0.826)	-0.321 (0.069)	-0.094 (0.609)
Peak flow L/min	-0.004 (0.981)	-0.267 (0.134)	-0.308 (0.086)
QOL	0.263 (0.074)	-0.093 (0.603)	0.081 (0.658)
Sputum neuts	-0.123 (0.495)	**0.482 (0.023)**	0.363 (0.116)
Blood neuts	-0.258 (0.080)	-0.020 (0.914)	
CRP	-0.097 (0.517)	**0.368 (0.038)**	
BSI score			0.021 (0.909)
Live PsA PCR	Exacerbation	Post Exacerbation	Clinical stability
FEV_1/L	-0.133 (0.368)	-0.317 (0.064)	-0.095 (0.594)
FEV_1 % pred	0.014 (0.926)	**-0.398 (0.018)**	-0.022 (0.901)
Peak flow L/min	-0.126 (0.394)	-0.251 (0.147)	-0.129 (0.466)
QOL	0.178 (0.225)	-0.078 (0.650)	0.082 (0.646)
Sputum neuts	-0.084 (0.649)	**0.563 (0.006)**	0.391 (0.089)
Blood neuts	-0.029 (0.874)	-0.159 (0.355)	
CRP	-0.011 (0.941)	**0.338 (0.047)**	
BSI score			0.091 (0.609)

FEV_1; Forced expiration value in 1 second, L; litre, QOL; quality of life questionnaire, ¥;log cells/g, *; log CFU/ml, §;10^9/L, Data for Blood total WCC, neutrophil count and serum CRP not available for clinical stability. BSI; Bronchiectasis severity index, , *p*-values in bold are significant if <0.05, r; Spearman's rho coefficient

7.4.3.4 Correlations between changes in clinical and microbiological outcomes between the beginning of a pulmonary exacerbation, the end of pulmonary exacerbation and clinical stability

The difference between clinical and microbiological parameters were calculated between the beginning of IV antibiotics and the end of IV antibiotics for a pulmonary

exacerbation by subtracting the exacerbation values from the post-exacerbation values. Therefore, a positive change would suggest an increase (i.e. lung function parameters and quality of life score) and a negative change would reflect a reduction (i.e. *P. aeruginosa* load, sputum neutrophils, blood neutrophils and serum CRP) between the beginning and end of pulmonary exacerbation.

The Sputum neutrophil count change between the beginning and end of IV antibiotics was positively correlated with *P. aeruginosa* load measured by culture (r=0.547 *p*=0.013) and serum CRP (r=0.572, *p*=0.011). Changes in *P. aeruginosa* load measured by culture and PCR were positively correlated, (r=0.682, *p*<0.001, Table 7-8). No other correlations were found for changes across a pulmonary exacerbation.

In addition, differences between clinical and microbiological parameters were calculated between the beginning of IV antibiotics for a pulmonary exacerbations and clinical stability by subtracting the exacerbation values from the clinical stability values.

The quality of life score difference between clinical stability and pulmonary exacerbation was correlated positively with *P. aeruginosa* load measured by culture (r=0.463, *p*-0.010). In addition, the difference between sputum neutrophils (r= 0.491, *p*=0.033) and *P. aeruginosa* load measured by culture (r=0.566, *p*=0.001) were positively correlated with *P. aeruginosa* load measured by PCR.

Table 7-8. Correlations between changes in clinical and microbiological outcomes between the beginning of a pulmonary exacerbation, the end of pulmonary exacerbation and clinical stability.

Post-Pre	Spearman's Correlation Coefficient, r (*p*-value)			
Variable	PsA culture*	Sputum neuts¥	PsA PCR*	CRP
FEV_1 L	-0.04 (0.796)	-0.111 (0.652)	-0.045 (0.797)	-0.048 (0.755)
FEV_1 % pred	-0.016 (0.931)	-0.200 (0.411)	-0.066 (0.706)	-0.038 (0.808)
Peak flow L/min	0.018 (0.922)	-0.305 (0.204)	-0.005 (0.979)	0.069 (0.657)
QOL	-0.199 (0.266)	-0.360 (0.119)	-0.269 (0.113)	-0.093 (0.546)
PsA culture load*		**0.547 (0.013)**	**0.682 (<0.001)**	0.112 (0.548)
Sputum neuts¥			0.344 (0.138)	**0.572 (0.011)**
Live PsA PCR*				-0.171 (0.326)
Stability-Pre				
Variable	PsA culture*	Sputum neuts¥	PsA PCR*	
FEV_1 L	0.262 (0.162)	-0.042 (0.864)	-0.145 (0.413)	
FEV_1 % pred	0.261 (0.163)	-0.044 (0.859)	-0.141 (0.426)	
Peak flow L/min	0.101 (0.595)	-0.009 (0.972)	-0.240 (0.171)	
QOL	**0.463 (0.010)**	-0.055 (0.825)	0.160 (0.365)	
PsA culture load*		0.318 (0.185)	**0.566 (0.001)**	
Sputum neuts¥			**0.491 (0.033)**	

FEV_1; Forced expiration value in 1 second, L; litre, QOL; quality of life questionnaire, ¥;log cells/g, *; log CFU/ml, § 10^9/L, neuts; neutrophils, Bacterial load; total bacterial load measured on blood agar, PsA; *P. aeruginosa*. Data for serum CRP not available for clinical stability.
Changes are calculated as Post exacerbation outcome minus the exacerbation outcomes and clinical stability outcome minus the exacerbation outcome. Therefore, a positive result suggests an improvement in lung function parameters and quality of life score and a reduction in *P. aeruginosa* load, sputum neutrophils, blood neutrophils and serum CRP.

7.4.3.5 Exploring differences in lung function and quality of life between the beginning and end of pulmonary exacerbation with relevant microbiology

For the participants who isolated *P. aeruginosa* within 12 months (n=32) or 4 weeks (n=25) of the study pulmonary exacerbation, there was no significant difference of lung function change between the beginning and end of pulmonary exacerbation (Table 7-9). In addition, participants who isolated *Haemophilus influenzae* (n=3) or a respiratory virus (n=10) at pulmonary exacerbation had no differences in lung function or quality of life.

In the sub-groups of participants who were known to be chronically colonised with *P. aeruginosa* (n=27), the peak flow declined between the beginning and end of IV antibiotics; -8.64L/min (SD±: 39.78) compared to those who were not colonised; 51.38 L/min (SD±: 111.86, *p*=0.016). There were no differences in lung function or quality of life in participants who were chronically colonised with *P. aeruginosa* over 5 years (n=14) compared to those were chronically colonised under 5 years (n=13).

Table 7-9. The changes of lung function and quality of life parameters between completion of IV antibiotics and the beginning of IV antibiotics for a pulmonary exacerbation with relevant hospital microbiology

Variable	FEV_1*	*p*-value	PEFR*	*p*-value	QOL*	*p*-value
Chronic PsA						
No	268.57 (±751.56)		51.38 (±111.86)		30.94 (±23.53)	
Yes	-18.80 (± 167.66)	0.069	-8.64 (±39.78)	**0.016**	22.15 (±14.45)	0.125
Years col						
<5 yr	12.73 (± 153.43)		-2.55 (± 32.76)		23.08 (±17.85)	
>5 yr	-43.57 (± 179.68)	0.416	-13.43 (± 45.16)	0.509	21.41 (±11.79)	0.781
PsA<12m						
No	267.33 (± 705.10)		49.27 (±114.37)		32.18 (±24.48)	
Yes	37.42 (± 425.08)	0.175	4.00 (±64.68)	0.093	23.21 (±16.03)	0.138
PsA< 4w						
No	248.70 (±722.84)		42.87 (±114.10)		31.29 (±19.59)	
Yes	-23.91 (± 160.36)	0.084	-5.35 (±27.13)	0.055	21.45 (±18.61)	0.084
Virus§						
No	126.49 (±593.03)		19.32 (±93.77)		26.32 (±19.56)	
Yes	54.44 (±164.71)	0.722	16.44 (±39.86)	0.929	26.06 (±20.46)	0.971
H.influ§						
No	73.02(±457.71)		15.14 (±79.72)		26.03 (±20.16)	
Yes	676.67 (±1243.88)	0.058	70.67 (±163.97)	0.282	29.67 (±3.70)	0.759

PsA; Pseudomonas aeruginosa, H. influ; Haemophilus influenzae, * calculated as post exacerbation value minus exacerbation value, described as mean value with standard deviation in brackets, §; isolation of organism at exacerbation, Virus; No; n=40, Yes; n=10, Haemophilus influenza, No; n=47, Yes;n=3. Chronic PsA No; n=23, Yes; n=27. PsA<12m; Pseudomonas aeruginosa isolated from sputum within 12 months of study exacerbation, No; n=18, Yes; n=32. Years colonised <5 years; n=13 , over 5 years; n=14. PsA< 4 weeks; Pseudomonas aeruginosa isolated from sputum within 4 weeks of study exacerbation, No; n=25, Yes; n=25. *p*-value derived by paired t-test and is significant if <0.05.

7.4.3.6 Differential cell counts in sputum at the beginning of pulmonary exacerbation, end of pulmonary exacerbation and at clinical stability

The median proportion of neutrophils in the sputum significantly decreased from the beginning of a pulmonary exacerbation (99.5%, IQR: 97 to 99.75) to the end of a pulmonary exacerbation (96.38%, IQR 91.69 to 99.25, *p*=0.012, Table 7-10). In addition, the median proportion of squamous cells (0.44%, IQR: 0 to 1.6) and viable cells (92.7, IQR: 73.68 to 95.92) decreased significantly from the beginning to the end

of the pulmonary exacerbation (2.55% IQR: 0.66 to 6.17, p=0.002 and 77.35%, IQR: 62.96 to 86.23, p=0.007, respectively, Table 7-10).

There was no difference between the median proportion of the other differential cell counts in the sputum between the beginning of pulmonary exacerbation and clinical stability.

The squamous cell proportion remained under 5% and therefore indicated a lower respiratory tract sputum sample with minimal oral mucosal contamination [372]. The proportion of viable cells ranged from 77.35 to 92.7% indicating sample sufficiency [373].

Table 7-10. Differential cell analysis of sputum at the start and end of a pulmonary exacerbation.

	Before antibiotics		After antibiotics		
Cell type	Median (%)	IQR	Median (%)	IQR	*p*-value
Neutrophils	99.5	97.00 to 99.75	96.38	81.69 to 99.25	**0.012**
Macrophages	0.25	0 to 1.50	2.5	0.24 to 15.30	0.054
Eosinophils	0.24	0 to 0.50	0.50	0.25 to 2.25	0.106
Lymphocytes	0	0 to 0	0	0 to 0	0.305
Epithelial	0	0 to 0.50	0.13	0 to 0.75	0.172
Squamous cells	0.44	0 to 1.60	2.55	0.66 to 6.17	**0.002**
Viable cells	92.7	73.68 to 95.92	77.35	62.96 to 86.23	**0.007**
	Before antibiotics		Clinical Stability		
Cell type	Median (%)	IQR	Median (%)	IQR	*p*-value
Neutrophils	99.5	97.00 to 99.75	98.88	89.5 to 99.25	0.747
Macrophages	0.25	0 to 1.50	0.34	0 to 6.44	0.824
Eosinophils	0.24	0 to 0.50	0.75	0.25 to 1.00	0.130
Lymphocytes	0	0 to 0	0	0 to 0	0.317
Epithelial	0	0 to 0.50	0.13	0 to 1.00	0.293
Squamous cells	0.44	0 to 1.60	0.40	0.17 to 1.47	0.653
Viable cells	92.7	73.68 to 95.92	90.27	84.11 to 92.40	0.8813

%; percentage, IQR; interquartile range

7.4.4 Summary of clinical and microbiological factors on clinical status

7.4.4.1 After treatment with IV antibiotics for a pulmonary exacerbation

After antibiotic treatment for a pulmonary exacerbation, there was a reduction in systemic inflammation biomarkers such as blood white cell count, blood neutrophil count, serum CRP, sputum neutrophil count and an improvement in the quality of life score which have been described previously [374]. There were no significant changes in pulmonary function but it is well described that lung function spirometric measures are not reliable indicators of exacerbation or response to treatment in bronchiectasis patients, unlike cystic fibrosis [375, 376].

The quantitative bacterial load measured by culture (*P. aeruginosa* and total bacterial load) did not change between the beginning and end of a pulmonary exacerbation which has also been previously recognised [189, 377]. However, the amount of *P. aeruginosa* sputum positivity from both hospital and quantitative microbiology significantly reduced which is consistent with previous data [378]. Interestingly, viable cells measured by PCR showed a reduction in *P. aeruginosa* load between the beginning of a pulmonary exacerbation and after treatment. This phenomenon is consistent with our CF cohort in Chapter 3, whereby the *P. aeruginosa* load measured by PCR reduced significantly after antibiotic treatment whilst there was no notable difference using culture. As previously discussed, this discrepancy is likely due to differences in methodology whereby enumeration of colonies are subject to individual operator error compared to universally validated PCR techniques.

7.4.4.2 Pulmonary exacerbation compared to clinical stability

The main differences between the beginning of a pulmonary exacerbation and clinical stability were a higher quantitative bacterial load measured by culture (total and *P. aeruginosa*) at clinical stability and an improvement of the quality of life score. In addition, the number of sputum samples positive for *P. aeruginosa* increased from pulmonary exacerbation to clinical stability. It has been shown previously that

bacterial load returns to high levels after suppression from antibiotic treatment and remains elevated over time during clinical stability [379].

7.4.4.3 Lung function

The absolute FEV_1 at the beginning of exacerbation negatively correlated with the patient age. Peak flow negatively correlated with age and positively correlated with BMI, which is consistent with previous data [380]. After treatment with IV antibiotics for a pulmonary exacerbation, the FEV_1 negatively correlated with sputum neutrophil count and blood neutrophil count, which has been previously recognised [381]. In addition, FEV_1 % predicted and the peak flow L correlated negatively with blood neutrophil count after exacerbation which has also been historically described [382]. A higher FEV_1 value after treatment for a pulmonary exacerbation was associated with a reduced *P. aeruginosa* load measured by culture and PCR (p=0.043 and p=0.018, respectively). Whilst it is recognised that a reduced bacterial load correlates with a reduction in systemic and localised inflammatory markers, studies in CF have showed that although exacerbations are not caused by an increase in *P. aeruginosa* burden, lung function improvements correlate with a reduction in *P. aeruginosa* load [383, 384].

At clinical stability, the total bacterial load in sputum correlated negatively with both the FEV_1/L and peak flow measurements. It has been shown previously that high airway bacterial loads are associated with airway and systemic inflammation and greater risk of subsequent exacerbation [79, 385, 386], and a cross-sectional negative correlation between total bacterial load and lung function has been previously described [379].

Overall, in this cohort, peak flow seemed to be the most consistent measurement of lung function improvement. This has been reflected in previous studies which have showed peak flow may be more reliably associated with clinical status compared to FEV_1 in non-CF bronchiectasis [370, 387].

7.4.4.4 Disease severity

A higher blood neutrophil count after exacerbation correlated with a higher bronchiectasis severity score. It has been noted previously that elevated inflammation markers such as white blood cell count and in particular blood neutrophil count are associated with the extent of disease severity at clinical stability [388]. In addition, we found a higher BSI score correlated positively with a higher total bacterial load at clinical stability which has been recently reported [379].

7.4.4.5 Quality of life scores

The quality of life scores consistently reflected clinical response showing an improvement from the beginning of pulmonary exacerbation to both after exacerbation and clinical stability. An increase in quality of life score at clinical stability compared to pulmonary exacerbation correlated with an increase in *P. aeruginosa* bacterial load. One explanation for this finding is bacterial load often recovers to baseline in the absence of systemic antibiotic therapy when patients are in a clinically stable state (16).

7.4.4.6 Microbiology and inflammation

Between the beginning and end of a pulmonary exacerbation, an increase in sputum neutrophils correlated with an increase in *P. aeruginosa* load measured by culture. In addition, between a pulmonary exacerbation and clinical stability, an increase in sputum neutrophils correlated with increase *P. aeruginosa* load measured by PCR.

After pulmonary exacerbation, the amount of sputum and blood neutrophils correlated negatively with FEV_1/L. For participants who were colonised with *P. aeruginosa*, there was a significant decline in peak flow L/min after exacerbation compared to those who were not.

7.4.4.7 Summary

In our cohort, the quality of life scores seemed to be the most consistent measurement at pulmonary exacerbation and clinical stability that correlated with clinical improvement. In addition, a lower blood and sputum neutrophil count and *P. aeruginosa* load (culture and PCR) after treatment for a pulmonary exacerbation reflected a higher lung function and may be useful measures to detect clinical response to antibiotics.

7.5 Exploring time to next pulmonary exacerbation and death

7.5.1 Time to next exacerbation from the end of initial exacerbation (visit 2)

The follow up period from consent of participants until the end of the study period (30/11/2020) ranged from 16.5 to 27.5 months. Out of 47 participants that were seen at the end of pulmonary exacerbation, 36 participants (76.6%) had a subsequent exacerbation before the end of the study period on 30/11/2020. The median interval to subsequent exacerbation from the end of initial exacerbation was 85.5 days (IQR: 51 to 164).

Multivariable modelling using a cox proportional hazard regression analysis was performed with adjustment for age and gender as *a priori* confounding factors and lung function (measured as FEV_1/L) as a measure of disease severity. As discussed in the methodology Chapter 6, proportional hazard assumptions were confirmed.

As shown in Table 7-11, the variables that were independently associated with time to next exacerbation were; an older age; (HR; 1.05, 95%CI: 1.01 to 1.10), male gender; (HR; 2.87, 95%CI: 1.36 to 6.07), FEV_1/L at the end of a pulmonary exacerbation; (HR; 0.41, 95%CI: 0.22 to 0.77), current smokers; (HR; 4.68, 95%CI: 1.12 to 19.50), exceeding more than 3 exacerbations in the last 12 months; (HR: 2.16, 95%CI; 1.06

to 4.38), chronic colonisation of non-mucoid *P. aeruginosa*; (HR; 2.41, 95%CI: 1.13 to 5.14) and blood neutrophil count; (HR; 1.12, 95%CI: 1.03 to 1.21).

Table 7-11. Time to next pulmonary exacerbation from end of initial exacerbation

Variable		HR	p-value	95% CI
Age		1.05	**0.029**	1.01;1.10
Male		2.87	**0.005**	1.36;6.07
Female		1.00		
Lung function at end of exacerbation°	FEV_1/L	0.41	**0.006**	0.22;0.77
BMI	0.99	0.857	0.93;1.06	
Co-morbidities	COPD	1.72	0.181	0.78;3.81
	Asthma	0.78	0.534	0.35;1.72
	IHD	0.74	0.502	0.30;1.80
	Pulm HTN	0.76	0.799	0.09;6.20
	Diabetes	3.57	0.064	0.93;13.73
Smoking status	Current	4.68	**0.034**	1.12;19.50
	Ex-smoker	1.30	0.503	0.60;2.84
	Passive	0.40	0.379	0.05;3.12
No. of exacerbations in last 12 months	>3	2.16	**0.032**	1.07;4.38
	≤ 3	1.00		
Years diagnosed	>4.25 years	1.57	0.358	0.60;4.09
	≤4.25 years	1.00		
PsA colonised	Any	1.75	0.145	0.82;3.71
	Mucoid	0.78	0.545	0.35;1.75
	Non-mucoid	2.41	**0.023**	1.13;5.14
Years colonised	>5 years	0.76	0.558	0.31;1.88
	≤5 years	1.00		
Colomycin nebulisers	N=6	0.63	0.402	0.22;1.85
Azithromycin	N=19	1.32	0.458	0.64; 2.72
Sputum bacterial Load (log_{10} CFU/g)	Total load	1.00	0.997	0.71; 1.41
	PsA load	0.97	0.698	0.86;1.10
	Live PsA PCR	0.97	0.669	0.84;1.12
Neutrophil count	Sputum*	0.93	0.889	0.36;2.45
	Blood [a]	1.12	**0.006**	1.03;1.21

Multivariable cox regression modelling (adjusted for age, gender, FEV_1/L)
°;HR per unit increase of FEV1/L and Peak flow L/min, PsA; Pseudomonas aeruginosa load, Total load; total bacterial load. IHD; ischaemic heart disease, Pulm;pulmonary * HR per unit increase in log_{10} neut/g, [a];HR per unit increase in 10^9/L, current smokers; 4 were male (80%) and 1 was female (20%).

7.5.2 Time to death from the start of a pulmonary exacerbation (visit 1)

Out of 50 participants recruited at the start of a pulmonary exacerbation, 12 (24%) died in the study period until censoring on 30/11/2020. As previously stated, 2 participants died between the beginning and end of a pulmonary exacerbation, 5 died between the end of a pulmonary exacerbation and clinical stability, and a further 5 died after visit 3 (clinical stability visit). Therefore, to capture all deaths, the study time to death was calculated as the date of consent to either date of death or until censoring (30/11/2020). The median interval to death (n=12) was 88 days (IQR: 31 to 221).

Again, multivariable modelling using a Cox proportional hazard regression analysis was performed with adjustment for age and gender as *a priori* confounding factors. In addition, the baseline lung function used to predict the bronchiectasis severity index (measured as FEV_1 % predicted at clinical stability) was used as a measure of disease severity. . As discussed in the methodology Chapter 6, proportional hazard assumptions were confirmed.

As presented in Table 7-12, the variables that were independently associated with time to death were; older age; (HR; 1.07, 95%CI: 1.01 to 1.14), FEV_1 % predicted at baseline; (HR; 0.96, 95%CI: 0.92 to 0.99); current smokers; (HR; 11.30, 95%CI: 1.19 to 107.16) and over 5 years chronic colonisation with *P. aeruginosa*; (HR; 0.09, 95%CI: 0.01 to 0.82). In addition, co-morbidities such as pulmonary hypertension; (HR; 15.57, 95%CI: 2.79 to 87.08) and diabetes (HR; 8.07, 95%CI: 1.09 to 59.94).

Table 7-12. Time to death from start of initial exacerbation

Variable		HR	p-value	95% CI
Age		1.07	**0.041**	1.00;1.14
Male		1.59	0.445	0.48;5.28
Female		1.00		
Lung function at baseline°	FEV_1 % pred	0.96	**0.021**	0.92;0.99
BMI		0.92	0.247	0.80;1.06
Co-morbidities	COPD	1.14	0.854	0.28;4.70
	Asthma	0.28	0.239	0.04;2.30
	IHD	1.37	0.710	0.26;7.11
	Pulm HTN	15.57	**0.002**	2.79;87.08
	Diabetes	8.07	**0.041**	1.09;59.94
Smoking status	Current	11.30	**0.035**	1.19;107.16
	Ex-smoker	1.27	0.742	0.30;5.35
	Never	1.00		
No. of exacerbations in last 12 months	>3	1.82	0.340	0.53;6.29
	≤ 3	1.00		
Years diagnosed	>4.25 years	0.69	0.594	0.17;2.75
	≤4.25 years	1.00		
PsA colonised	Any	2.03	0.302	0.53;7.80
	Mucoid	0.80	0.747	0.20;3.14
	Non-mucoid	2.59	0.144	0.72;9.27
Years colonised	>5 years	0.09	**0.032**	0.01;0.82
	≤5 years	1.00		
Colomycin nebulisers	N=6	1.09	0.937	0.13;9.43
Azithromycin	N=19	1.00	0.994	0.27;3.61
Sputum bacterial§	Total load	1.56	0.134	0.87;2.79
Load (log_{10} CFU/g)	PsA load	1.13	0.213	0.93;1.38
	Live PsA PCR	1.16	0.225	0.91;1.50
Neutrophil count §	Sputum*	1.55	0.646	0.24;10.05
	Blood ª	1.10	0.096	0.98;1.24

Multivariable cox regression modelling (adjusted for age, gender, FEV_1 % predicted) °;HR per unit increase of FEV_1 % predicted, PsA; Pseudomonas aeruginosa load, Total load; total bacterial load. IHD; ischaemic heart disease, Pulm; pulmonary * HR per unit increase in log_{10} neut/g, ª;HR per unit increase in 10^9/L, § at the start of a pulmonary exacerbation, current smokers; 4 were male (80%) and 1 was female (20%).

7.5.3 Summary of time to next exacerbation and death

7.5.3.1 Factors associated with both exacerbation and mortality risk

In this section, we have described the independent factors that were associated with time to next exacerbation and death. An older age, lower lung function and current smokers were all associated with a higher exacerbation and mortality risk.

It is well recognised that disease severity in non-CF bronchiectasis is linked to older age and a lower FEV1/L [6]. In fact, these measures are incorporated in the validated severity scores such as BSI and FACED score which predict mortality and hospitalisation/exacerbation rate [389]. In addition, cigarette smoke is a major trigger for pulmonary exacerbations in a variety of pulmonary diseases, including COPD and bronchiectasis [390]. The link between smoking history and mortality in bronchiectasis has also been previously described [391]. Recently Sin *et al* showed individuals with bronchiectasis, air flow limitation and ever smokers had a higher risk of all-cause mortality [392].

7.5.3.2 Factors associated with pulmonary exacerbation risk

In our data, male gender was associated with almost a three times greater exacerbation risk over the study period of 27.5 months. This is an interesting finding as it is common for females to be associated with increased risk of exacerbations, poorer clinical outcomes, and worse survival in non-CF bronchiectasis [371, 393], due to chronic lung inflammation contributing to greater tissue damage and therefore disease severity in females [394]. Our data showed that lung function was higher in males compared to females at the beginning and end of a pulmonary exacerbation and clinical stability. However, one possible explanation for a higher exacerbation risk in males may be due to a higher prevalence of current smokers (80% in males versus 20% in females), which we have showed is an independent predictor of exacerbation and mortality risk in our data.

In addition, these data demonstrate that a higher quantitative blood neutrophil count at the end of treatment for a pulmonary exacerbation was associated with a 12% increased risk of exacerbation. This data is consistent with our clinical outcomes whereby blood neutrophils predicted a clinical response to IV antibiotic therapy. As blood neutrophils are a marker of systemic inflammation, and this itself is associated with poorer outcomes in non-CF bronchiectasis, it is feasible that a higher neutrophil count after treatment may subsequently predict a further pulmonary exacerbation [79, 395].

An interesting new finding in our data is that chronic colonisation with non-mucoid *P. aeruginosa* was associated with a two and a half time greater risk of exacerbations in a sub-group analysis. In many studies, the association of chronic *P. aeruginosa* infection with disease severity, mortality and exacerbations does not differentiate between mucoid or non-mucoid strains [6, 396, 397].

Many of the hypotheses regarding the natural history and behaviour of *P .aeruginosa* in the airways of people with non-CF bronchiectasis have been extrapolated from CF. It is well known that *P .aeruginosa* undergoes evolutionary changes in CF with colonial morphological alterations along with antibiotic susceptibility changes [161]. The switch to a mucoid phenotype (due to an overproduction of alginate) in CF is a marker of the transition to chronic infection and worse outcomes [398, 399]. The switch to a mucoid phenotype is associated with higher persistence of *P. aeruginosa* in the airways coupled with decreased virulence factor production and quorum-sensing deficiency [400]. In addition, compared with non-mucoid strains, the mucoid phenotype is associated with a lower success of eradication treatment, but these studies were in CF [401, 402]. The biology and implications of persistent infection of *P. aeruginosa* are less well described in non-CF bronchiectasis [400]. The available data does suggest that there are similar adaptive mechanisms of *P .aeruginosa* present in the non-CF bronchiectasis lung but there are also key differences with CF [106, 400, 403]. We have also showed in our data that the duration of chronic colonisation was longer with mucoid compared to non-mucoid strains. Nevertheless,

despite some adaptive similarities between CF and non-CF bronchiectasis isolates, longitudinal data are currently too limited to draw adequate comparisons in the biology *of P. aeruginosa* from the two diseases.

In non-CF bronchiectasis, Serrano *et al* reported that the mucoid phenotype was associated with worse severity outcomes [404] and Czaja *et al* demonstrated that mucoid strains were associated with worse lung function, but there were no differences in exacerbation frequencies or hospitalizations [405]. Indeed, a recent study by Luo *et al* showed that the presence of virulence genes *exoU* and *pldA* in mucoid isolates were associated with greater exacerbation risk in non-CF bronchiectasis [406]. However, out of 147 mucoid isolates, the prevalence of *pldA* and *exoU* were 18.5% and 11.1% respectively, confirming virulence factors are present in only a minority of mucoid strains.

It is important to consider that data can also be confounded by multiple mutations from a single isolate, along with differing phenotypes and genotypes all being present in the lung environment of the same individual. Indeed, people with CF and non-CF bronchiectasis can be infected with multiple strains of *P. aeruginosa* that will not be apparent using routine microbiological culture techniques in the clinical setting [191]. Therefore, the discrimination of mucoid versus non-mucoid colonisation by standard hospital culture may not be truly reflective of the strains currently infecting the individual in question.

Nevertheless, chronic colonisation with non-mucoid *P. aeruginosa* isolates may be associated with increased virulence factor production compared to mucoid strains and therefore it is biological plausible that it caused a higher exacerbation risk in our cohort.

7.5.3.3 Factors associated with increased mortality

As described above, older age, lower lung function and current smokers were associated with increased mortality. In addition, we found that a larger number of

years of chronic colonisation with *P. aeruginosa* was associated with a reduction in mortality risk.

As previously discussed, this finding may be due to long-term adaptation of *P. aeruginosa* isolates in the lung environment in people with non-CF bronchiectasis. It has been shown by Woo *et al* that there is a trend towards reduced virulence factor production in *P. aeruginosa* non-CF bronchiectasis isolates over time but this has not directly been linked to clinical outcomes [407]. In CF, chronic infection is linked to biofilm production and immune evasion rather than an inflammatory or cytotoxic process seen in acute infections [408]. In addition, long-term dominance of infection with *P. aeruginosa* may compete against other harmful pathogens such as *Staphylococcus aureus* and *Haemophilus influenzae* through microbial interactions [205, 409-412].

Another possible explanation for this finding may be that detection of early colonisation of *P. aeruginosa* in the clinical setting may allow eradication attempts with extensive systemic and inhaled antimicrobial regimes by clinicians. Indeed, this is recommended in both European and national non-CF bronchiectasis guidelines [68, 413]. This in turn would lead to increased patient and microbiological surveillance and may favour short-term clinical outcomes and therefore impact survival.

Finally, we saw that diabetes and pulmonary hypertension were associated with an eight and fifteen times increased risk of mortality in our cohort, respectively. Pulmonary hypertension itself is a known complication of non-CF bronchiectasis and the association with increased mortality is well-recognised [414-416]. This is most likely due to hypoxaemia and patients having more extensive lobar involvement [416] in non-CF bronchiectasis.

Diabetes itself is associated with a greater all-cause mortality in people with or without pulmonary disease [417]. In addition, diabetes is associated with an increased mortality in people with CF and COPD [418, 419]. Although the exact reasons are unclear, it is hypothesised that there is an additional inflammatory burden from

diabetes [418]. In fact, our data are consistent with findings in the Bronchiectasis Aetiology Comorbidity Index (BACI) whereby pulmonary hypertension and diabetes were evaluated as strong predictors of five-year mortality in non-CF bronchiectasis [366].

7.6 Discussion

This chapter describes the results of an observational cohort study investigating the influence of clinical, microbiological and inflammatory factors on clinical status and disease progression in non-CF bronchiectasis.

We describe that a reduction in local and systemic markers of inflammation are useful measures to predict treatment response in non-CF bronchiectasis. In addition, quality of life questionnaires and peak flow measurements of lung function are the most consistent clinical measurements that reflect clinical status in this cohort. A higher total bacterial load at clinical stability correlated with a worse lung function, quality of life and bronchiectasis severity score. Finally, several novel factors associated with mortality and exacerbation risk have been described such as duration of colonisation and non-mucoid strains of *P. aeruginosa*.

7.6.1 Strengths

The strengths of this study include a well-defined cohort that is representative of hospitalized participants experiencing severe pulmonary exacerbations. Detailed baseline and phenotypical characteristics were collected at the start and end of a pulmonary exacerbation and at clinical stability. The methodology for this observational study (described in chapter 5) was robust and followed a strict study protocol with all the laboratory measures gathered in a standardized manner. Standard consensus definitions of a pulmonary exacerbation and chronic colonisation status of individuals were used. Lastly, the follow up period from consent until censoring (range 16.5 to 27.5 months) allowed accurate modelling for exacerbation interval and mortality risk.

7.6.2 Limitations

There are several limitations in our study. Despite the consensus definitions of pulmonary exacerbations used in this study, symptoms are often subjective by both the patient and the clinician. In addition, treatment initiation, regimes and length were decided upon the discretion of a clinician. These factors mean that there is likely to be considerable variability across the cohort with treatment response. Our cohort represents a severe spectrum of disease whereby patients were hospitalised or requiring IV antibiotics for a pulmonary exacerbation.. In addition, our cohort had a higher prevalence of smoking exposure and COPD as a co-morbidity meaning the data may be biased and not representative of the local bronchiectasis population as a whole.

It is important to note that although the median duration between commencement of IV antibiotics and recruitment was 0.5 days, samples including sputum should have been taken prior to antibiotic therapy. This may explain the many discrepancies in our data and future studies need to ensure that the antibiotic effect on the sputum microbiota is minimalised. The exploratory analysis of numerous clinical and microbiological factors in this cohort may mean significant results are result of multiple hypothesis testing. In addition, as only 50 participants were recruited, some analyses was likely to be underpowered to show a true effect and further study with an adequate power calculation and research question is needed. Lastly, investigating the aetiology of bronchiectasis, microbiological colonisation status and other baseline demographics were reliant on historical records and these may be subject to inaccuracy.

7.6.3 Summary

Despite limitations, this study does reflect current clinical practice and although there is considerable heterogeneity in non-CF bronchiectasis populations, the robust protocols and definitions used in our study have permitted confidence in the comparability of our data. In this chapter, we have described several clinical and

microbiological factors that are associated with adverse clinical outcomes, disease severity, time to exacerbation and mortality.

Chapter 8. Investigating the association between urinary inflammatory biomarkers and clinical status in cystic fibrosis and non-cystic fibrosis bronchiectasis

8.1 Introduction

This chapter describes the investigation of urinary samples collected from 61 adults with cystic fibrosis (CF) and 44 adults with non-CF bronchiectasis. Urinary samples were tested for five inflammatory biomarkers: (i) Neutrophil Gelatinase Associated Lipocalin, (ii) Clara Cell 16 (CC16), (iii) Tissue Inhibitor of Metalloproteinase 1, (iv) Fibrinogen and (v) C-Reactive Protein (CRP).

8.1.1 Background

Bronchiectasis encompasses a range of clinical disorders that result in permanent dilatation of the bronchi and is characterised clinically by a chronic productive cough and recurrent respiratory infections. Bronchiectasis can be caused by both inherited conditions and acquired aetiologies [1]. Traditionally, distinction is made between the autosomal recessive condition cystic fibrosis (CF) and other causes of non-cystic fibrosis bronchiectasis.

Pulmonary exacerbations in both CF and non-CF bronchiectasis are defined as a deterioration in respiratory symptoms such as cough, sputum production or purulence and breathlessness, with or without systemic upset, necessitating antibiotics [69, 317]. Pulmonary exacerbations are associated with increased mortality, significant lung function decline and reduced quality of life [6]. Severe pulmonary exacerbations

requiring hospital admissions and antibiotics are also costly to the health service. However, despite the existence of exacerbation definitions, diagnostic challenges still remain in the healthcare setting. There are a notable lack of therapeutic end-points for pulmonary exacerbations [420].

Pulmonary exacerbations in CF and non-CF bronchiectasis are primarily driven by pulmonary infection and neutrophilic airway inflammation, which leads to progressive lung damage [421]. Systemic markers of inflammation such as blood neutrophilia and C-Reactive Protein (CRP) are increased in people with CF and non-CF bronchiectasis experiencing a pulmonary exacerbation [422]. However, the diagnostic utility of using urinary inflammatory markers in pulmonary exacerbations is currently not known [423]. Urinary concentrations of Desmosine, a break down product of elastin, have been shown to decrease significantly following treatment for a pulmonary exacerbation in CF [424]. However, Desmosine has not been investigated at clinical stability and no correlations have been observed with clinical parameters such as lung function or inflammatory markers [424]. A non-invasive urinary marker of inflammation could potentially aid early diagnosis of pulmonary exacerbations and facilitate early medical intervention which may avoid hospitalisation and limit further lung damage.

A urinary lateral flow assay provided by Mologic Ltd is a urine-based multi-biomarker self-test kit that has been validated in a chronic obstructive pulmonary disease pulmonary (COPD) exacerbation cohort [425]. The *in vitro* diagnostic self-test kit has been designed to detect five biomarkers indicative of infection and inflammation in the urine of COPD patients: Neutrophil Gelatinase Associated Lipocalin (NGAL), Clara Cell 16 (CC16), Tissue Inhibitor of Metalloproteinase 1 (TIMP-1), Fibrinogen and C-Reactive Protein (CRP). This point-of-care test allows a result in 10 minutes that is connected to a mobile application, promoting patient self-management.

NGAL is an antimicrobial peptide elevated in respiratory conditions such as COPD and CF that has been shown to inhibit bacterial growth and enhance collagen matrix degradation [426, 427]. CC16 is an anti-inflammatory protein secreted by the Clara cells in the distal respiratory epithelium and has been proposed as a biomarker of lung

epithelial injury in various lung diseases, including CF [428]. TIMP-1 is elevated in COPD exacerbations and has been indicated in airway pathogenesis and remodelling in various lung diseases including COPD and pulmonary fibrosis [429, 430]. Both Fibrinogen and CRP are acute phase proteins and are systemically raised in people with CF and non-CF bronchiectasis experiencing pulmonary exacerbations [431, 432].

8.1.2 Aims

COVID-19 IMPACT STATEMENT

Due to the COVID-19 pandemic, the non-CF bronchiectasis feasibility study for quorum sensing molecules was delayed by the time of writing this thesis. As such, further analysis was performed to explore the role of novel urinary inflammatory molecules in this cohort.

We performed a proof to concept study to determine the diagnostic potential of these five urinary biomarkers in CF and bronchiectasis cohorts using urinary lateral flow devices. In this chapter, our aims were to explore if urinary levels of NGAL, CC16, TIMP-1, Fibrinogen and CRP:

- are detectable in the urine of adults with CF and non-CF bronchiectasis
- reflect clinical status across a pulmonary exacerbation
- are associated with clinical outcomes such as lung function, local or systemic inflammation and time to next exacerbation

8.2 Methods

8.2.1 Study design and participants

Urine samples which had been collected and stored during two biomarkers studies in CF (Research Ethics Committee; 09/H0407/1) [279, 280] and non-CF bronchiectasis (Research Ethics Committee; 18/WM/0125, methodology described in Chapter 4) were

analysed. In summary, random urine samples (25ml) were obtained and frozen at -80°C from participants within 72 h of the start and end of intravenous (IV) antibiotic therapy for a pulmonary exacerbation, and at clinical stability. CF samples were collected and between 2011 and 2013. Non-CF bronchiectasis samples were collected and stored between 2019 and 2020.

The CF cohort consisted of adults with CF attending two UK CF centres. An acute pulmonary exacerbation was defined according to Rosenfeld criteria [317]. The bronchiectasis samples were obtained from adults attending a single UK centre. Similarly, a pulmonary exacerbation was defined according to Hill *et al* [69].

For both cohorts, clinical stability was defined as the absence of an acute pulmonary exacerbation in the preceding 4 weeks. Baseline demographic and clinical data were collected for both cohorts.

8.2.2 Urinary lateral flow assay multi-biomarker analysis

A Manual DataReader Software Cube and 280 urinary lateral flow devices were provided by Mologic Ltd. These devices measure five urinary biomarkers: Neutrophil Gelatinase Associated Lipocalin (NGAL), Clara Cell 16 (CC16), Tissue Inhibitor of Metalloproteinase 1 (TIMP-1), Fibrinogen and C-Reactive Protein (CRP). The devices were designed and calibrated using a COPD cohort and were not modified for these analyses [433]. The Cube Reader provided quantitative and qualitative evaluation of lateral flow assays, using a mobile measuring device. *DataReader Software* was used to extract the measurement data from the Cube.

Urine samples were thawed at room temperature and 650µl of urine was inserted onto each urinary lateral flow device using sterile calibrated pipettes. At 10 minutes, the device was placed into the cube reader. Cube readings were converted into concentrations (ng/mL) using the standard curves for each biomarker on the MyAssays ® Ltd online software [434]. The standard curves for each of the biomarkers are

provided in Table 8-1. Concentration ranges within the standard measurements for each biomarker were as follows: NGAL: 1.024-250ng/mL; CC16: 1000-4.096ng/mL; TIMP-1: 0.8192-200ng/mL; Fibrinogen: 4.096-1000ng/mL and CRP: 0.256-25ng/mL (Table 8-1).

8.2.3 Statistical analysis

For primary analysis, Wilcoxon signed-rank tests were used to compare concentrations of NGAL, CC16, TIMP-1, Fibrinogen and CRP between paired urinary samples in individuals at the start of a pulmonary exacerbation and then after the patient had received systemic antibiotics.

The secondary analysis compared concentrations of NGAL, CC16, TIMP-1, Fibrinogen and CRP at the start of a pulmonary exacerbation with available matched urinary samples at clinical stability. Changes in detectable concentrations were quantified by calculating log fold changes by: calculating the ratio of matched (i) post exacerbation levels over exacerbation levels and (ii) stability levels over pre-exacerbation levels and subsequently log transforming the data (log2).

If statistical differences were found between levels of the potential biomarkers and exacerbation status, further analyses were performed. Associations between biomarker concentrations at pulmonary exacerbation, after treatment and clinical stability were explored with forced expiratory volume in 1 second (FEV1) percent predicted, quantitative sputum (log_{10} cells/g) and blood (10^9/L) neutrophil count using Two-sample t-tests or Mann-Whitney U tests.

Data were divided into above the median and below or equal to the median concentration value (ng/ml) for each of the measured biomarkers at each time-point (start of exacerbation, after treatment and clinical stability). This was necessary to minimise discrepancies in any detectable value measured outside of the standard

curve, as these devices have not been calibrated in non-CF bronchiectasis or CF cohorts.

A multivariable Cox proportional hazards model, adjusted for age, gender and lung function was used to explore associations between the median levels of a biomarker of interest at clinical stability to time to next pulmonary exacerbation. Data were censored to the end of the study period on 30/11/2020. Proportional hazard assumptions were confirmed. All analyses were performed in Stata SE15 statistical software (Texas, USA).

8.3 Results

In the CF cohort, 61 matched urine samples at the beginning and end of IV antibiotics and 27 matched urine samples at clinical stability were analysed. In the non-CF bronchiectasis cohort, out of 44 participants, 43 urine samples were available at the beginning of a pulmonary exacerbation and 41 were available at the end of IV antibiotics for analysis (due to sample volume). In addition, 37 matched samples were available at clinical stability.

Serum CRP and blood neutrophil count were available for the non-CF bronchiectasis cohort and median levels decreased significantly from the beginning of a pulmonary exacerbation; 12.5 mg/L (interquartile range IQR:0.5-23.5) and 6.5 10^{9}/L (IQR:5.0-11.0) respectively, to 5.0 mg/L (IQR:0.0-12.0, p=0.019) and 5.7 10^{9}/L (IQR:4.4-8.2, p=0.006, Table 8-1), respectively. The baseline demographics for the CF and non-CF bronchiectasis cohort are summarised in Table 8-2.

Table 8-1. Standard curves for; Neutrophil Gelatinase Associated Lipocalin, Clara Cell 16, Tissue Inhibitor of Metalloproteinase 1, Fibrinogen and C-Reactive Protein.

Standard	NGAL	Cube units	CC16	Cube units	TIMP1	Cube units	Fib	Cube units	CRP	Cube units
	Conc ng/ml		Conc ng/ml		Conc ng/ml		Conc ng/ml		Conc ng/ml	
1	250	219.0	1000	10.2	200	282.9	1000	153.1	25	241.9
2	100	180.1	400	25.9	80	254.9	400	122.7	10	194.8
3	40	115.7	160	57.9	32	207.7	160	77.5	4	129.7
4	16	62.7	64	100.7	12.8	144.2	64	43.1	1.6	71.9
5	6.4	29.9	25.6	135.0	5.12	86.1	25.6	24.6	0.64	37.2
6	2.56	14.5	10.24	151.5	2.048	42.3	10.24	15.1	0.256	18.1
7	1.024	10.0	4.096	156.6	0.8192	19.2	4.096	10.5		
8	0	7.3	0	160.0	0	5.3	0	8.0	0	5.0
Conc; concentration, ng/ml; nanograms per millitre										

The percentage of biomarkers detected in the urine ranged from 100% for CC16 and TIMP in both cohorts, to 59% for Fibrinogen in CF. The number of detected biomarkers and the number obtained outside the standard concentration curves are summarised in Table 8-3.

Table 8-2. Baseline demographics of CF and bronchiectasis cohort

Variable*	Cystic fibrosis		
Mean age, years: (range)	30.5 (17; 59)		
Gender: female (%)	26 (44.1)		
Mean BMI, kg/m^2: (SD)	21.9 (±3.7)		
	Pre (n=61)	Post (n=61)	Stability (n=27)
Mean $FEV_{1,}$ L: (SD)	1.7 (0.7)	2.0 (0.8)	1.7 (0.6)
Mean % predicted FEV_1: (SD)	47.2 (16.9)	53.1 (18.7)	48.7 (14.5)
Mean sputum neutrophils cells/g: (SD)°	7.1 (0.4)	6.9 (0.4)	7.2 (0.5)
Variable*	Bronchiectasis		
Mean age, years: (range)	69.0 (43.7; 83.2)		
Gender: female (%)	26 (59.1)		
Mean BMI, kg/m^2: (SD)	23.6 (±6.6)		
	Pre (n=44)	Post (n=44)	Stability (n=37)
Mean $FEV_{1,}$ L: (SD)	1.1 (± 0.6)	1.2 (±0.8)	1.2 (±0.8)
Mean % predicted FEV_1: (SD)	43.4 (±21.7)	45.4 (± 24.0)	47.3 (±23.1)
Mean sputum neutrophils cells/g: (SD)°	7.1 (±0.5)	6.8 (±0.7)	7.1 (±0.6)
Median serum CRP: (IQR)	12.5 (0.5-23.5)	5 (0-12)	*p*=0.019§
Median blood neutrophils: (IQR)	6.5 (5.0-11.0)	5.7 (4.4-8.2)	*p*=0.006§

n; number of participants, FEV_1; Forced expiratory volume in 1 second, L; Litre, % pred; percent predicted. °; (log_{10} cells/g), CRP measured as mg/L, blood neutrophils measured as 10x ^{9}/L.*;variable measured at pulmonary exacerbation, SD; standard deviation, §; *p*-value derived from Wilcoxon signed-rank test.

Table 8-3. Detected concentrations and number of detected outside of the standard curve.

	NGAL	CC16	TIMP-1	Fibrinogen	CRP
CF					
No detected: N (%)					
At exacerbation, n= 61	60 (98)	61 (100)	61 (100)	36 (59)	51 (84)
After treatment, n=61	60 (98)	61 (100)	61 (100)	41 (67)	38 (62)
Clinical stability, n=27	27 (100)	27 (100)	27 (100)	19 (70)	26 (96)
Out of range: N (%)					
At exacerbation, n= 61	1 (2)	7 (11)	27 (44)	5 (14)	17 (33)
After treatment, n=61	0	31 (51)	26 (43)	1 (3)	16 (42)
Clinical stability,n=27	1 (4)	1 (4)	13 (48)	1 (5)	9 (35)
Bronchiectasis	NGAL	CC16	TIMP-1	Fibrinogen	CRP
No detected: N (%)					
At exacerbation, n=43*	42 (98)	43 (100)	43 (100)	26 (60)	37 (86)
After treatment, n=41°	41 (100)	41 (100)	41 (100)	34 (83)	29 (71)
Clinical stability, n=37	37 (100)	37 (100)	37 (100)	29 (78)	30 (81)
Detected but out of range: N (%)					
At exacerbation	0	19 (44)	23 (53)	0	6 (16)
After treatment	1 (2)	21 (51)	18 (44)	1 (3)	9 (31)
Clinical stability	0	11 (30)	15 (41)	1 (3)	12 (40)

No detected; Number detected (concentrations over zero), *; 1 sample had insufficient urinary volume for analysis at the start of a pulmonary exacerbation, °;only 41 samples out of 44 were available for analysis. detected but out of range: concentrations detected outside of the standard curve, all concentrations measured as ng/ml.

8.3.1 Changes in median urinary biomarker concentrations measured at the start and end of treatment for a pulmonary exacerbation

Median concentrations of all five measured biomarkers at the beginning and end of a pulmonary exacerbation are shown in Table 8-4. Urinary CC16 levels significantly increased following treatment for a pulmonary exacerbation in both CF and non-CF bronchiectasis cohorts from 467.7 ng/ml (IQR: 201.3-738.5) and 796.3 ng/ml (IQR: 315.6-1724.0), respectively, to 1044.0 ng/ml (IQR: 490.3-1740.0, $p<0.001$) and 1102.0 ng/ml (IQR: 531.7-1948.5, $p=0.04$), respectively. Urinary CRP levels significantly decreased following treatment for a pulmonary exacerbation in the CF cohort from 0.4 ng/ml (IQR: 0.1-1.1) to 0.1 ng/ml (IQR: 0-0.4, $p=0.013$). There was no significant decrease in urinary CRP following antibiotics in the non-CF bronchiectasis cohort and any other urinary inflammatory molecules in CF or non-CF bronchiectasis (Figure 8-1 and 8-2).

8.3.2 Changes in median urinary biomarker concentrations measured at the start of a pulmonary exacerbation compared to clinical stability

Median concentrations of all five measured biomarkers at the beginning of a pulmonary exacerbation and clinical stability are shown in Table 8-5. In the CF cohort, concentration of urinary CC16 was lower at stability; 248.5 ng/ml (IQR: 108.4-607.0) compared to the start of a pulmonary exacerbation; 467.7 ng/ml (IQR: 201.3-738.5, $p=0.042$).

In the non-CF bronchiectasis cohort, median values of CC16 and CRP were significantly lower at clinical stability; 317.1 ng/ml (IQR: 159.1-1211.0) and 0.2 ng/ml (IQR: 0.0-1.2), respectively, compared with the start of a pulmonary exacerbation; 796.3 ng/ml (IQR: 315.6-1724.0, $p=0.018$) and 0.56 ng/ml (IQR: 0.2-2.5, $p=0.002$) respectively. TIMP-1 was significantly higher at clinical stability; 1.5 ng/ml (IQR: 0.5-2.7) compared to at the start of a pulmonary exacerbation; 0.8 ng/ml (IQR: 0.3-2.8, $p=0.045$).

There were no significant associations between the concentrations of NGAL or fibrinogen with clinical status in either the CF or the non-CF bronchiectasis cohorts (Table 8-2, Figure 8-1 and 8-2).

A representation of the log mean concentration (ng/ml) trajectory of all 5 biomarkers is represented in Figure 8-3.

Table 8-4. Median levels of five urinary biomarkers at the start and end of treatment for a pulmonary exacerbation.

	Start exacerbation	After exacerbation	*p*-value
Cystic Fibrosis			
NGAL	25.2 (14.2-50.7)	30.5 (15.1-52.9)	0.618
CC16	467.7 (201.3-738.5)	1044.0 (490.3-1740.0)	**<0.001**
TIMP	1.0 (0.4-2.7)	1.2 (0.4-3.3)	0.821
Fibrinogen	2.4 (0-15.1)	14.7 (0-34.0)	0.065
CRP	0.4 (0.1-1.1)	0.1 (0-0.4)	**0.013**
Bronchiectasis			
NGAL	16.2 (4.7-36.9)	18.9 (8.8-38.6)	0.452
CC16	796.3 (315.6-1724.0)	1102.0 (531.7-1948.5)	**0.039**
TIMP	0.8 (0.3-2.8)	1.1 (0.3-3.1)	0.519
Fibrinogen	3.2 (0-15.9)	10.6 (5.1-49.3)	0.073
CRP	0.56 (0.2-2.5)	0.2 (0-1.4)	0.065

Concentrations are measured as nanograms per millilitre (ng/mL), NGAL-Neutrophil Gelatinase Associated Lipocalin, CC16- Clara Cell 16 (CC16), TIMP-1-Tissue Inhibitor of Metalloproteinase 1, CRP- C-Reactive Protein. Values are expressed as median concentration with interquartile range in brackets, p-value determined by Mann-whitney U tests and are bold if <0.05.

Table 8-5. Median levels of five urinary biomarkers at the start of a pulmonary exacerbation and clinical stability.

	Start exacerbation	Clinical Stability	*p*-value
Cystic Fibrosis			
NGAL	25.2 (14.2-50.7)	23.6 (10.2-40.3)	0.414
CC16	467.7 (201.3-738.5)	248.5 (108.4-607.0)	**0.042**
TIMP	1.0 (0.4-2.7)	0.9 (0.4-2.5)	0.501
Fibrinogen	2.4 (0-15.1)	5.1 (0-15.9)	0.415
CRP	0.4 (0.1-1.1)	0.3 (0.2-1.0)	0.058
Bronchiectasis			
NGAL	16.2 (4.7-36.9)	19.2 (4.7-38.6)	0.167
CC16	796.3 (315.6-1724.0)	317.1 (159.1-1211.0)	**0.018**
TIMP	0.8 (0.3-2.8)	1.5 (0.5-2.7)	**0.045**
Fibrinogen	3.2 (0-15.9)	17.7 (3.2-33.1)	0.069
CRP	0.56 (0.2-2.5)	0.2 (0.0-1.2)	**0.002**

Concentrations are measured as nanograms per millilitre (ng/mL), NGAL-Neutrophil Gelatinase Associated Lipocalin, CC16- Clara Cell 16 (CC16), TIMP-1-Tissue Inhibitor of Metalloproteinase 1, CRP- C-Reactive Protein. Values are expressed as median concentration with interquartile range in brackets, p-value determined by Mann-whitney U tests and are bold if <0.05.

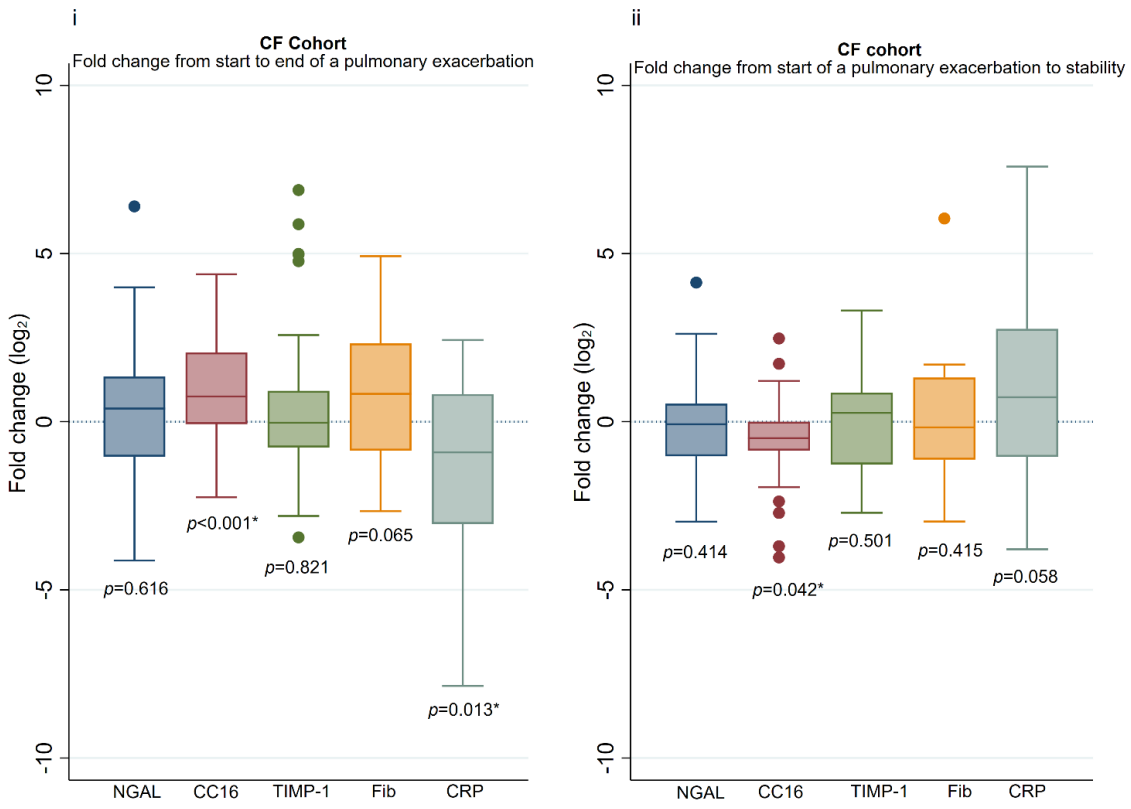

Figure 8-1. CF cohort: Fold changes in concentration of NGAL, CC16, TIMP-1, Fibrinogen and CRP levels (i) from the beginning of a pulmonary exacerbation to the end of a pulmonary exacerbation, (ii) from the beginning of a pulmonary exacerbation to clinical stability.

Figure legend: Box plots represent median and whiskers represent interquartile range, NGAL- Neutrophil Gelatinase Associated Lipocalin, CC16- Clara Cell 16 (CC16), TIMP-1-Tissue Inhibitor of Metalloproteinase 1, CRP- C-Reactive Protein, *p*-value determined by Mann-Whitney U tests.

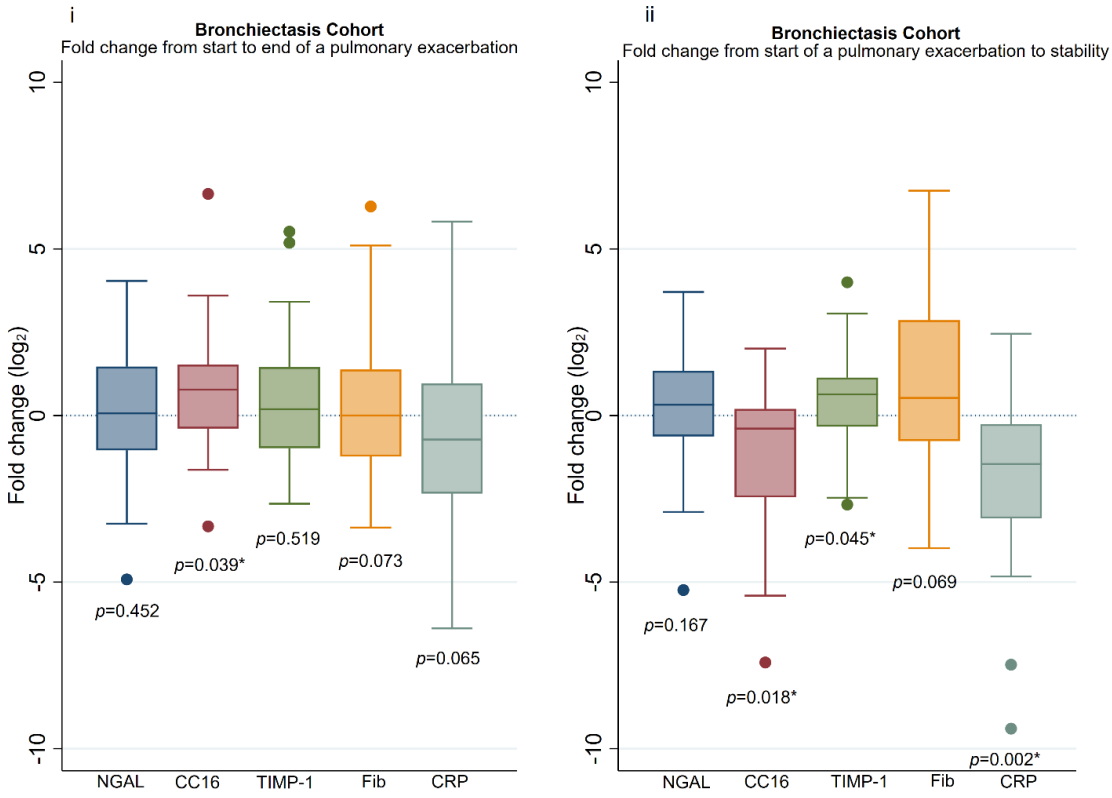

Figure 8-2. Non-CF bronchiectasis cohort: Fold Change in concentrations of NGAL, CC16, TIMP-1, Fibrinogen and CRP levels (i) from the beginning of a pulmonary exacerbation to the end of a pulmonary exacerbation, (ii) from the beginning of a pulmonary exacerbation to clinical stability.
Figure legend: Box plots represent median and whiskers represent interquartile range, NGAL- Neutrophil Gelatinase Associated Lipocalin, CC16- Clara Cell 16 (CC16), TIMP-1-Tissue Inhibitor of Metalloproteinase 1, CRP- C-Reactive Protein, *p*-value determined by Mann-Whitney U tests.

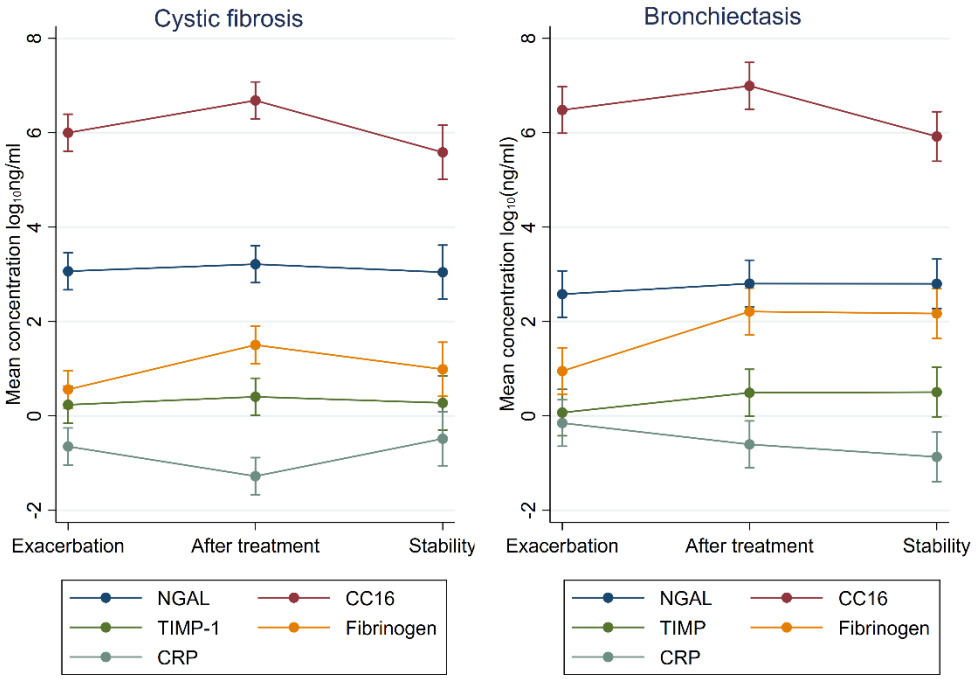

Figure 8-3. Log mean concentrations of NGAL, CC16, TIMP-1, Fibrinogen and CRP at acute exacerbation, after treatment and clinical stability.
Figure 3 legend. Mixed effected model of repeated measures represented as mean concentration (log_{10} ng/ml) to compare the trajectory of NGAL, CC16, TIMP-1, Fibrinogen and CRP.

8.3.3 Associations between levels of urinary CC16, TIMP-1 and CRP with FEV_1, sputum neutrophils and blood neutrophils

Further analyses were performed on the three biomarkers (CC16, TIMP-1 and CRP) which changed significantly with exacerbation status in the preliminary analyses.

High urinary CRP levels (above the median) were associated with a lower FEV_1 % predicted at the start of a pulmonary exacerbation in both the CF and non-CF bronchiectasis cohorts. Mean FEV_1 % predicted at beginning of a pulmonary exacerbation was 39.8% (SD±14.5) and 36.9% (SD±15.0) for above median CRP levels compared to 53.6% (SD±16.5, p=0.002) and 49.8% (SD±25.5, p=0.048) for below or equal to median CRP values, in the CF and non-CF bronchiectasis cohorts, respectively (Table 8-6, Figure 8-4).

Similarly, after treatment for a pulmonary exacerbation, urinary CRP levels measured above the median were associated with a lower FEV_1% predicted. Above median CRP levels were associated with an FEV_1 of 46.5% (SD±18.1) and 33.9% (SD±16.3) compared to FEV_1 of 58.8% (SD±17.4, p=0.011), and 54.9% (SD±26.6, p=0.007) for below or equal to the median values, in the CF and non-CF bronchiectasis cohort respectively (Table 8-6, Figure 8-4).

At the beginning of a pulmonary exacerbation in non-CF bronchiectasis, high urinary CRP levels were associated with a higher blood neutrophil count. Above median urinary CRP levels were associated with blood neutrophil counts of 7.75x 10^9/L (IQR: 5.2- 11.0) compared to 5.0x 10^9/L (IQR: 3.8- 6.4) for below or equal to the median urinary CRP levels (p=0.039, Table 8-7).

After treatment for a pulmonary exacerbation in non-CF bronchiectasis, high urinary CRP levels (above the median) were associated with a higher sputum and blood neutrophils levels compared to those with lower urinary CRP values. Above median urinary CRP values were associated with high sputum neutrophils counts (7.3 $\log_{10}$ cell/g (SD±0.70)) and higher blood neutrophil counts (6.8 x10^9/L (IQR: 5.2- 11.0) compared to those with lower urinary CRP levels (sputum neutrophil counts of 6.4 $\log^{10}$

cell/g (SD±0.6, *p*=0.024) and blood neutrophil counts of 5.0x10^9/L (IQR: 3.8- 6.4, *p*=0.025, Figure 8-5), respectively. No other associations were found between urinary CRP, CC16 or TIMP-1 levels and lung function or sputum and blood neutrophil counts (Table 8-6 and 8-7).

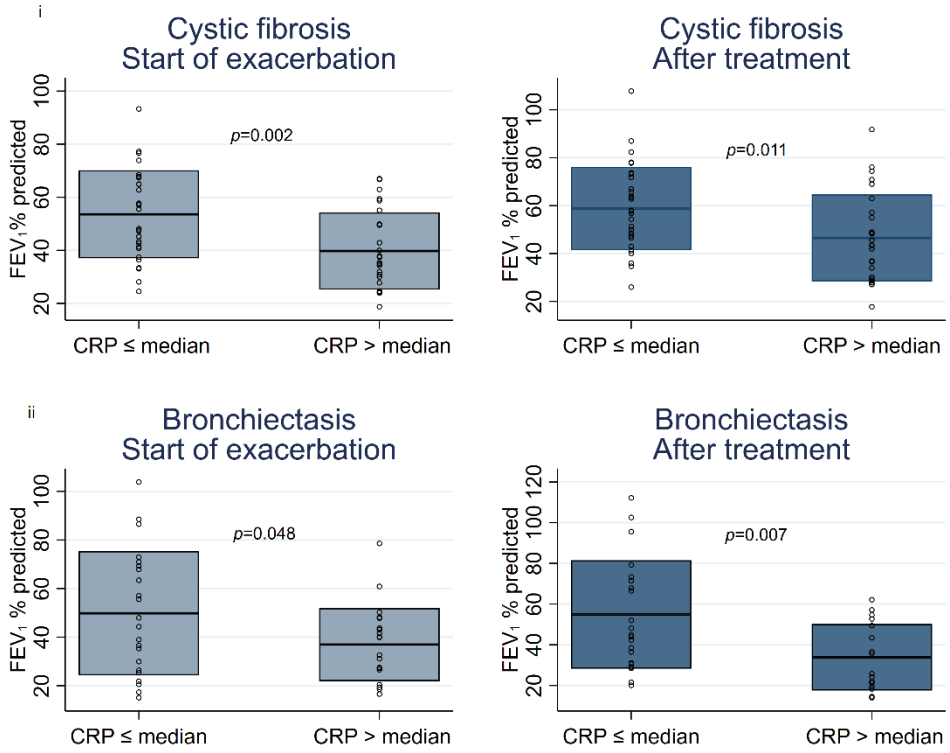

Figure 8-4. FEV_1 percent (%) predicted above or below/equal to the CRP median for CF and non-CF bronchiectasis at the start of a pulmonary exacerbation and after treatment for a pulmonary exacerbaion.
Figure 4 legend. Box line represents mean, box area represents +/- standard deviation. *p*-values derived from Two-sample t-tests. CRP concentration measured as ng/ml.

Table 8-6. Associations between above and below (or equal to) median levels of CC16, TIMP-1 and CRP with FEV_1 percent predicted at the start of an exacerbation, after treatment and stability.

Cystic Fibrosis			
Start exacerbation	FEV_1 % ≤ median	FEV_1 % > median	p-value
CC16	45.16 (±16.37)	49.44 (±17.78)	0.350
TIMP-1	45.97 (±13.09)	48.14 (±20.44)	0.634
CRP	**53.59 (±16.54)**	**39.75 (±14.53)**	**0.002**
Post exacerbation			
CC16	57.34 (±19.95)	46.54 (±14.59)	0.130
TIMP-1	52.49 (±22.36)	53.62 (±14.42)	0.821
CRP	**58.75 (±17.44)**	**46.51 (±18.13)**	**0.011**
Clinical stability			
CC16	44.86 (±12.82)	52.77 (±15.47)	0.159
TIMP-1	46.00 (±12.13)	51.53 (±16.60)	0.329
CRP	51.50 (±13.78)	45.62 (±15.10)	0.300
Bronchiectasis			
Start exacerbation	FEV_1 % ≤ median	FEV_1 % > median	p-value
CC16	42.88 (23.24)	43.97 (±20.31)	0.870
TIMP-1	42.92 (±18.92)	44.31(±24.59)	0.838
CRP	**49.82(±25.54)**	**36.93 (±15.04)**	**0.048**
Post exacerbation			
CC16	50.59 (±26.51)	39.44 (±21.57)	0.167
TIMP-1	40.99 (±19.02)	49.87(±29.63)	0.262
CRP	**54.91 (26.63)**	**33.86 (16.29)**	**0.007**
Clinical stability			
CC16	53.82 (±24.97)	39.11 (±18.99)	0.058
TIMP-1	43.61 (±20.38)	51.53 (±26.33)	0.312
CRP	50.36 (±26.45)	44.41 (±20.18)	0.449

CC16- Clara Cell 16 (CC16), TIMP-1-Tissue Inhibitor of Metalloproteinase 1, CRP- C-Reactive Protein, concentrations are measured as nanogram per millilitre. FEV_1% predicted represented as mean values and ±- represents standard deviation, *Values in bold are represented as $p<0.05$ and are determined by Two-sample t-tests.

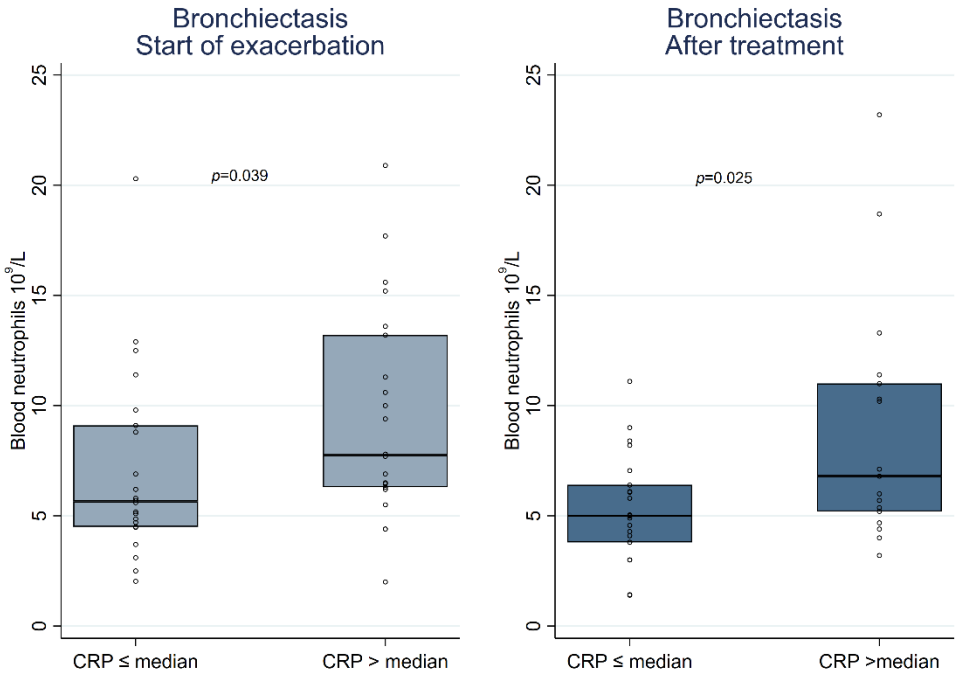

Figure 8-5. Blood neutrophils above or below/equal to the CRP median for non-CF bronchiectasis at the start of a pulmonary exacerbation and after treatment for a pulmonary exacerbation.
Figure 5 legend. Box line represents median, box area represents interquartile range, p-value derived from Mann-Whitney U test. CRP concentration measured as ng/ml.

Table 8-7. Associations between above and below (or equal to) median levels of CC16, TIMP-1 and CRP with blood and sputum neutrophils.

Cystic Fibrosis			
Start exacerbation	Sputum neut ≤ median	Sputum neut > median	p-value
CC16	7.17 (±0.35)	7.10 (±0.42)	0.507
TIMP-1	7.11 (0.39)	7.18 (±0.38)	0.536
CRP	7.08 (±0.43)	7.20 (±0.32)	0.246
Post exacerbation			
CC16	6.86 (0.41)	6.92 (0.37)	0.630
TIMP-1	6.93 (0.31)	6.84 (±0.45)	0.404
CRP	6.83 (±0.41)	6.95 (0.36)	0.272
Clinical stability			
CC16	7.32 (±0.51)	6.95 (±0.45)	0.125
TIMP-1	7.27 (±0.55)	7.03 (±0.45)	0.322
CRP	7.19 (±0.56)	7.12 (±0.49)	0.786
Bronchiectasis			
Start exacerbation	Sputum neut ≤ median	Sputum neut > median	p-value
CC16	7.24 (±0.43)	6.99 (±0.61)	0.225
TIMP-1	7.14 (±0.60)	7.08 (±0.52)	0.745
CRP	7.04 (±0.55)	7.15 (±0.54)	0.614
Post exacerbation			
CC16	6.48 (±0.53)	7.09 (±0.86)	0.109
TIMP-1	6.66 (0.51)	7.00 (±0.96)	0.372
CRP	**6.42 (0.61)**	**7.25 (±0.70)**	**0.024**
Clinical stability			
CC16	7.21 (±0.75)	7.05 (±0.55)	0.631
TIMP-1	7.15 (±0.72)	7.09 (±0.57)	0.842
CRP	6.99 (±0.58)	7.21 (±0.67)	0.495
Start exacerbation	Blood neut ≤ median°	Blood neut > median°	p-value
CC16	6.40 (4.68-9.85)	6.68 (5.35-12.25)	0.423
TIMP-1	6.3 (4.86-12.5)	7.1 (5.65-10.65)	0.742
CRP	**5.65 (4.5-9.1)**	**7.75 (6.3-13.2)**	**0.039**
Post exacerbation			
CC16	5.04 (4.29-7.05)	6.25 (4.9-8.4)	0.278
TIMP-1	5.94 (4.15-8.7)	5.29 (4.49-7.66)	0.715
CRP	**5.0 (3.8-6.4)**	**6.8 (5.2-11.0)**	**0.025**

CC16- Clara Cell 16 (CC16), TIMP-1-Tissue Inhibitor of Metalloproteinase 1, CRP- C-Reactive Protein, concentrations are measured as nanogram per millilitre. Sputum neutrophils are measured as $\log_{10}$ cells per gram and represented as mean values and ±- represents standard deviation, °;Blood neutrophils measured as 10^{9}/L and are represented as median and interquartile range in brackets. *Values in bold are represented as $p<0.05$ and are determined by paired t-tests or Mann-Whitney U tests for blood neutrophils.

8.3.4 Exploring time to next exacerbation from clinical stability in non-CF bronchiectasis

Data on time to next exacerbation from clinical stability was available in the non-CF bronchiectasis cohort only. Out of 37 participants who provided urine samples at clinical stability, 24 (64.9%) participants experienced an exacerbation before the end of the study period (30/11/2020). The median time to next exacerbation was 80 days (IQR: 30- 225, Table 8-8).

In the non-CF bronchiectasis cohort, the time to next exacerbation was a median of 51 weeks (IQR: 6-76) when urinary CC16 levels were above the median values, compared to 24 weeks (IQR: 11-72) when urinary CC16 levels were below or equal to the median values (HR; 0.2, 95%CI; 0.1-0.8, *p*=0.017, Figure 8-6, Table 8-8). In addition, the time to next exacerbation when urinary TIMP-1 levels were above the median was 15 weeks (IQR: 4-55), compared to 67 weeks (IQR:7-88) when urinary TIMP-1 levels were below or equal to the median value (HR; 6.0, 95%CI; 1.7- 21.4, *p*=0.006, Figure 8-6, Table 8-8). No associations were found for urinary CRP levels and time to next exacerbation.

Table 8-8. Time to next exacerbation from clinical stability (n=37) in non-CF bronchiectasis.

Variable		HR	*p*-value	95% CI
Age		1.01	0.783	0.96-1.05
Male		1.73	0.171	0.79-3.81
Female		1.00		
FEV_1/L		0.63	0.137	0.34-1.16
Exacerbation: n (%)	24 (64.9)			
No exacerbation: n (%)	13 (35.1)			
	Median days (IQR)	HR	*p*-value	95% CI
Time to next exacerbation	80 (30-225)			
CC16				
>Median	360.5 (43-533.5)	0.24	**0.017**	0.08-0.78
≤Median	171 (76-501)	1.00		
TIMP				
>Median	103 (26-387)	5.97	**0.006**	1.66-21.42
≤Median	469 (49-613)	1.00		
CRP				
>Median	229.5 (49-566)	0.78	0.564	0.34-1.80
≤Median	279 (40-501)	1.00		

Multivariable Cox proportional hazards model, adjusted for age, gender and lung function. Data censored to time to next exacerbation or end of study period- 30/11/2020. HR- Hazards ratio, 95%CI- 95% confidence interval, IQR- interquartile range., n- number of participants, FEV_1/L- forced expiration in second per litre. CC16- Clara Cell 16 (CC16), TIMP-1-Tissue Inhibitor of Metalloproteinase 1, CRP- C-Reactive Protein

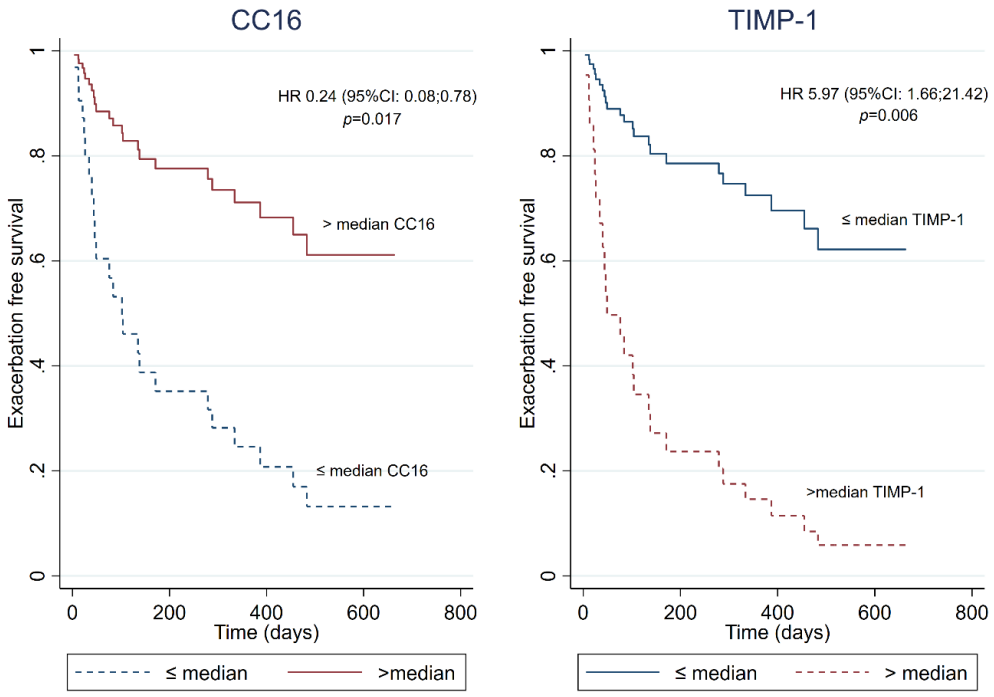

Figure 8-6. Cox proportional hazards regression graphs on above and below (or equal to) the median levels of CC16 and TIMP-1 on time to next exacerbation (adjusted for gender, age and FEV_1)
Figure 6 legend: Cox proportional hazard regression graphs adjusted for age, gender and FEV_1. CC16- Clara Cell 16 (CC16), TIMP-1- Tissue Inhibitor of Metalloproteinase 1, HR- Hazards ratio, 95%CI- 95% confidence interval

8.4 Discussion

This study is a proof of concept study that aims to determine if five urinary inflammatory markers measured using novel quantitative rapid lateral flow assays could be used to predict pulmonary exacerbation status in CF and non-CF bronchiectasis cohorts. We found all five inflammatory markers (NGAL, CC16, TIMP-1, Fibrinogen and CRP) were detected in the urine of adults with CF and non-CF bronchiectasis. Three of the 5 measured markers correlated with changes in clinical status in these cohorts.

Specifically, urinary CRP levels significantly declined from the start to the end of treatment for a pulmonary exacerbation in CF. Urinary CRP levels were significantly lower at clinical stability in the non-CF bronchiectasis cohort compared to at the start of

a pulmonary exacerbation. Urinary CC16 levels in both CF and non-CF bronchiectasis were consistently higher after a pulmonary exacerbation and lower at clinical stability. Urinary TIMP-1 levels in non-CF bronchiectasis cohort were higher at clinical stability compared to a pulmonary exacerbation. In addition, higher levels of urinary CRP in CF and bronchiectasis correlated with a lower lung function before and after exacerbation and higher sputum and blood neutrophils in non-CF bronchiectasis after treatment. Finally, we found that low urinary CC16 levels and high TIMP-1 levels were associated with a shorter time to next exacerbation in the non-CF bronchiectasis cohort.

CRP is an acute phase protein is increased in the blood of people with CF and non-CF bronchiectasis experiencing a pulmonary exacerbation [375]. However, there is no data on the role of urinary CRP measurements in these patient populations. Our data on urinary measures of CRP are consistent with our CRP systemic measurements and other studies, whereby a rise in CRP is often noted at the beginning of a pulmonary exacerbation compared to after treatment and at clinical stability [387, 435].

CC16 possesses anti-inflammatory and immunosuppressant properties [436, 437]. Serum or sputum assays of CC16 have often been used to assess lung injury in a variety of chronic pulmonary diseases but have rarely been assessed as a urinary assay [438]. Lower levels of CC16 have been found in the airways of lung transplant patients with bronchiolitis obliterans and smokers [439, 440]. Our data are consistent with these observations: urinary CC16 levels increased after treatment for a pulmonary exacerbation and high levels were associated with a longer time to next exacerbation. However, we did observe a lower median urinary CC16 level at stability compared to at the start of a pulmonary exacerbation, which was not expected. This finding contrasts with the findings of Laguna *et al* who found that sputum club cell secretory protein (CCSP) levels; another term for CC16, were lower at that the start of a pulmonary exacerbation compared to clinical stability in a CF cohort [441]. However, the Laguna study showed no significant correlations between sputum and blood CCSP concentrations and the study did not measure urinary levels. In addition, our cohort had

different baseline characteristics such as more severe pulmonary disease which may explain this discrepancy [441].

Conflicting data exist for TIMP-1, a tissue inhibitor of metalloproteases, in pulmonary disease, including cystic fibrosis. Animal and human studies support a role for the imbalance between MMPs and TIMPs in the pathogenesis of various pulmonary disorders through airway remodelling and fibrosis [442, 443]. Studies have showed the ratio of MMPs in relation to TIMP-1 in CF increases in acute pulmonary exacerbation [444, 445] and disease severity in non-CF bronchiectasis [193, 446]. However, another study showed serum expression of MMP-9 and TIMP-1 were significantly increased at clinical stability in those with moderate to severe disease compared to mild disease or the absence of lung function decline in adults and children with CF [447]. Furthermore, one study has showed that plasma levels of TIMP-1 greater than the median were associated with increased mortality in CF [448]. Rath *et al* showed serum TIMP-1 was a biomarker for CF associated liver disease and portal hypertension [449]. Our data also show the inconsistency with TIMP-1 levels and clinical status. Higher levels of TIMP-1 were associated with a shorter time to next exacerbation in the non-CF bronchiectasis cohort. However, levels were higher at stability compared during an exacerbation in the same cohort. This suggests that more research is needed to understand the role of this protein in pulmonary exacerbations examining both potential direct relationships and a mismatch ratio with MMPs.

8.4.1 Strengths

There are several strengths of the study. The magnitude of change of CRP and CC16 with change in clinical status is high and the consistent trend across two different disease cohorts provides confidence in the robustness of the observations. The use of a novel lateral flow urinary assay provided rapid semi-quantitative results on both individuals with CF and non-CF bronchiectasis. Urine samples were collected and stored in -80 degree freezers without delay and thawed only once for use in this study. To reduce multiple comparisons, we performed secondary analyses only on markers which were correlated with exacerbation status.

8.4.2 Limitations

There are several limitations that must be taken into consideration. The urinary lateral flow devices have been validated in a COPD cohort. The expected range of urinary signal levels is not known and may be subject to significant inter-individual variations. In addition, the collection of urine samples used a random “catch” method that may be associated with random error in the concentration of molecules being measured. Some of the urinary molecules; such as CC16, are excreted by the kidneys and therefore interpretation with renal function and urinary creatinine is needed in future. Despite the biomarker measurements being detectable, some fell outside the standard curve range, meaning the precise concentration could not be accurately determined. This meant that analysis was limited to binary outcomes.

The clinical phenotypes of CF and non-CF bronchiectasis, in particular, are heterogeneous and results may not be applicable to larger populations. In addition, the CF cohort was recruited more than ten years prior to this analysis and prior to the widespread use of personalised modulatory therapy, which may affect lung inflammation [450]. In the CF cohort, data on blood neutrophil counts and time to next exacerbation from clinical stability were missing. Lastly, further validation is needed in a prospective study design to determine the expected levels of variability at an individual level and should focus on three promising biomarkers only to limit multiple hypothesis testing.

8.4.3 Summary

In summary, this chapter describes a proof of concept study that reports levels of urinary markers (CRP and CC16) change between the start and end of treatment for pulmonary exacerbations, and at clinical stability in CF and non-CF bronchiectasis patients. In addition, urinary levels of TIMP-1 and CC16 have potential to highlight people at clinical stability who are at risk of a future exacerbation. Further larger prospective validation studies are warranted to determine if these urinary biomarkers may be useful clinically at an individual level. This approach has the potential to lead to

a home surveillance programme with non-invasive point of care testing promoting patient self-management in future.

Chapter 9. Exploring the clinical impact of pro-inflammatory cytokines and urinary biomarkers on *P. aeruginosa* status in a cohort of patients with non-CF bronchiectasis

9.1 Introduction

This chapter describes the investigation of sputum and urinary pro-inflammatory cytokines and their correlation with a pulmonary exacerbation or response to treatment and *P. aeruginosa* status in a non-CF bronchiectasis cohort.

9.1.1 Background

Persistent neutrophilic airway inflammation and chronic bacterial infection play a fundamental role in the pathogenesis of non-CF bronchiectasis and lead onto adverse outcomes such as lung function decline (Figure 9-1) [451]. The specific triggers of pulmonary exacerbations for non-CF bronchiectasis remain unknown, although several theories exist including overgrowth of certain pathological organisms or complex interactions between microbial communities and host immune response in the lung environment [452, 453].

The host defence and the inflammatory response in bronchiectasis are based on a complex cytokine network which facilitates the activation and recruitment of neutrophils into the airways [454]. The severity of the inflammatory response depends on an interplay between anti-inflammatory and pro-inflammatory cytokines and it is this imbalance which may cause local or systemic pathology [455]. Neutrophil-predominant acute inflammation is associated with the antigen-specific Th17 T cell pathway, whose primary function is the clearance of extracellular fungal and bacterial pathogens [456]. However, although the main effect of the Th17 pathway is the defence against micro-organisms, it may also be associated with airway damage in

people with bronchiectasis. Furthermore, recent evidence suggests that the airway inflammatory response triggered by bacterial stimulation continues to persist even after infection is controlled [457].

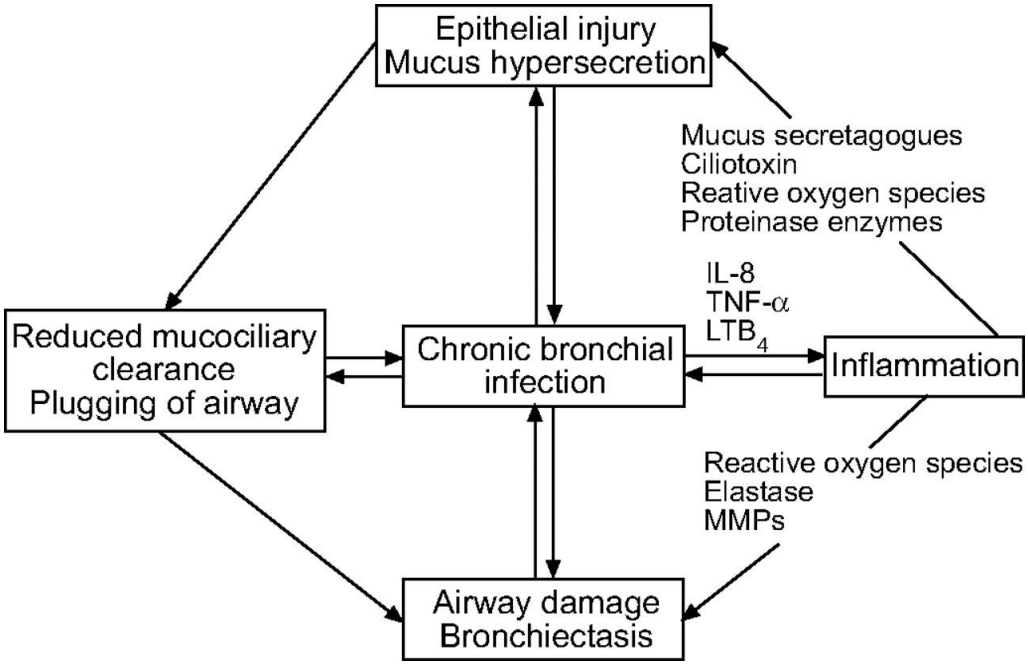

Figure 9-1. Schematic representation of a vicious circle of events which occurs during chronic bronchial infection. Image provided by Fuschillo *et al* [454]. Reproduced with permission of the © ERS 2021: European Respiratory Journal 31 (2) 396-406; DOI: 10.1183/09031936.00069007 Published 31 January 2008.
Schematic representation of a vicious circle of events which occurs during chronic bronchial infection. IL: interleukin; TNF: tumour necrosis factor; LT: leukotriene; MMP: matrix metalloproteinase

9.1.2 Overview of pro-inflammatory cytokines

The airways in non-CF bronchiectasis are abundant with neutrophils and pro-inflammatory mediators such as myeloperoxidase, interleukin (IL)-6 and IL-8, tumour necrosis factor (TNF)-alpha [142]. The Th17 pathway cytokines (IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α) have significantly higher levels in broncho-alveolar fluid in non-CF bronchiectasis patients compared to normal controls [108]. However, pulmonary TNF-α and IL-8 are suggested as the main neutrophil chemotactic mediators in non-CF bronchiectasis [385, 458]. Previous studies have reported higher sputum levels of

IL-8 have been associated with more severe non-CF bronchiectasis disease, increased sputum neutrophils and frequent exacerbations [459-461]. The concentrations of TNF-α and IL-8 in sputum supernatant have been shown to significantly reduce between the beginning of a pulmonary exacerbation and after treatment [378, 462]. In addition, IL-1b, IL-6, IL-8, and TNF-α have increased concentrations in the sputum of non-CF bronchiectasis patients compared to normal controls and a higher bacterial load is associated with more intense airway inflammation [385, 463]. Interestingly, in the same study, there were no correlations between systemic and bronchial inflammatory mediators suggesting that the inflammatory process may be compartmentalised in non-CF bronchiectasis airways [385].

Recently, Menendez *et al* reported a prospective observational study comparing systemic blood pro-inflammatory cytokines in exacerbation and stable groups in non-CF bronchiectasis [464]. IL-1β, IL-8, IL-17a and TNF-α levels increased similarly on days 1 and 5 in severe and non-severe pulmonary exacerbations compared to IL-6. However, on day 30, IL-17a, IL-8, and IL-6 levels were only increased for severe exacerbations compared to TNF-α [464]. Levels of IL-17a were higher in in patients who had chronic colonisation plus the acute isolation of *P. aeruginosa* and severe exacerbations were associated with higher systemic levels of IL-6a, IL-8 and IL-17a.

Furthermore, Chen *et al* described higher levels of IL-17a and IL-23 in broncho-alveolar lavage from non-CF bronchiectasis patients compared to normal controls [465]. However, IL-17a was not associated with any clinical measures or airway microbiology unlike IL-8 which was associated with *P. aeruginosa* load.

To date, there has been no investigation of urinary pro-inflammatory cytokines as non-invasive biomarkers of clinical status and *P. aeruginosa* bacterial load in people with non-CF bronchiectasis.

9.1.3 Aims

COVID-19 IMPACT STATEMENT

Due to the COVID-19 pandemic, the non-CF bronchiectasis feasibility study for quorum sensing molecules was delayed by the time of writing this thesis. As such, further analysis was performed to explore the role pro-inflammatory cytokines in this cohort.

The aims of this chapter are:

- To explore the correlation of the six Th17 pathway pro-inflammatory cytokines (IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α) in sputum and urine samples in people with bronchiectasis
- To explore if the levels of urinary pro-inflammatory cytokines reflect clinical status in our bronchiectasis observational cohort study
- To explore the associations between urinary inflammatory markers and *P. aeruginosa* quantitative load and colonisation status in bronchiectasis.

9.2 Methods

9.2.1 Study design and participants

The methodology of the bronchiectasis cohort exacerbation study including: study design, recruitment, sample collection and analysis has been described previously in Chapter 5. However, additional details of the methodology and statistical analysis are described below. In summary, sputum and random urine samples (25ml) were obtained and frozen at -80°C from participants within 72 h of the start and end of intravenous (IV) antibiotic therapy for a pulmonary exacerbation, and at clinical stability.

9.2.2 Cytokine analysis

9.2.2.1 Sample preparation

Six pro-inflammatory cytokines released by the respiratory epithelium (IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α) were analysed from available sputum and urine samples obtained before and after treatment for a pulmonary exacerbation and clinical stability.

Out of 50 participants experiencing a pulmonary exacerbation, 20 sputum supernatants and 19 matched urinary samples were available for cytokine analysis. Aliquots of sputum supernatant and urine were originally stored in -80°C freezers and thawed at room temperature on the day of processing.

9.2.2.2 Reagent preparation

All reagents were left to warm to room temperature before use. Firstly, the wash buffer was prepared by adding 20mL of wash buffer concentrate to 480mL of distilled water. All standards were reconstituted in Calibrator Diluent RD6-52 according to the Certificate of Analysis for reconstitution volumes. Standard 1 was prepared using 100µl of each standard in 700 µl of Calibrator Diluent. A serial dilution was then performed producing a 3-fold dilution where standard 1 served as the high standard. The calibrator diluent served as the blank.

9.2.2.3 Processing

Cytokines were analysed using a multiplex immunoassay system. The human magnetic luminex assay plates with 6 analytes (IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α) were provided by biotechne®.

Samples (50 µl) were pipetted to a 96-well plate coated with monoclonal antibodies for incubation for 2 h under room temperature. Following rinsing of the plate with buffer solution, the solution containing magnetic microbeads was added for further incubation. Following thorough rinsing with buffer solution, streptavidin-phycoerythrin

was added to incubate for 30 min. Stop solution (1M sulfuric acid) was used to terminate the reaction.

The concentration of inflammatory mediators were then assessed using a BioPlex reader (Bio-Rad, Hemel Hempstead, UK). The concentrations of the inflammatory mediators were recorded as pg/ml and the standard curves for each analyte are described in Table 9-1.

Table 9-1. Standard curves for IL-1 beta/IL-1F2 IL-6 IL-8/CXCL8 IL-17/IL-17A IL-23 TNF-alpha.

Sputum pg/ml						
Standard	IL-1 β	IL-6	IL-8	IL-17a	IL-23	TNF-α
1	4425.11	1070.9	1057.76	3532.15	31180.35	1831.93
2	1463.55	361.83	357.25	1110.18	10399.76	608.02
3	500.75	117.13	115.08	387.82	3469.19	203.91
4	159.46	37.81	40.42	128.66	1071.28	67.82
5	55.61	14.43	12.84	43.95	466.99	22.55
6	18.1	4.27	4.39	13.9	109.6	7.54
Urine pg/ml						
Standard	IL-1 β	IL-6	IL-8	IL-17a	IL-23	TNF-α
1	4420.01	1070.04	1060	3283.32	31099.99	1830
2	1473.29	356.53	355.33	1082.1	10411.3	615.17
3	491.24	119.12	116.43	362.35	3416.3	200.57
4	163.6	39.47	39.28	122.46	1160.56	67.1
5	54.6	13.26	13.24	40.21	387.9	23.28
6	18.19	4.4	4.33	13.43	125.53	7.42

pg/ml; pictograms per millilitre, IL; interleukin, IL-1b; IL-1 beta, TNF-α;TNF-alpha.

9.2.3 Statistical analysis

9.2.3.1 Pro-inflammatory cytokines in sputum and urine

The correlation of paired sputum and urinary pro-inflammatory cytokines were analysed using Spearman's correlation co-efficients.

Wilcoxon signed-rank tests were used to compare the levels of IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α initially between the start of a pulmonary exacerbation with concentrations after a course of treatment with systemic antibiotics. Further analysis then compared concentrations at the start of a pulmonary exacerbation and clinical stability.

If statistical differences were found between the levels of sputum or urinary cytokines and exacerbation status, further analyses were performed with urinary cytokines and clinical outcomes. Data were divided into binary measures of above the median and below or equal to the median concentration value (pg/ml) for each of the measured analytes at the three time-points (at the start of exacerbation, after treatment and clinical stability). This was necessary to minimise discrepancies in any detectable value measured outside of the standard curve.

Associations between urinary cytokine concentrations (above or below or equal to the median concentration for each individual cytokine of interest) and forced expiratory volume in 1 second (FEV_1)/L, bronchiectasis severity index (at clinical stability), quantitative sputum (log_{10} cells/g) and blood (10^9/L) neutrophil count were performed using Two-sample t-tests or Mann-Whitney U tests.

9.2.3.2 Urinary cytokines and urinary inflammatory molecules with P. aeruginosa status

Data exploring urinary cytokines and inflammatory markers with *P. aeruginosa* burden were assessed at clinical stability as participants would not have received antibiotics for at least 4 weeks.

The correlation of 5 urinary inflammatory markers (NGAL, CC16, TIMP-1, Fibrinogen and CRP) and 6 pro-inflammatory cytokines (IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α) were compared with *P. aeruginosa* quantitative load measured by culture and PCR using Spearman's rank correlations.

The concentration of both the urinary inflammatory markers and cytokines were then compared using Mann-Whitney U tests with four binary measures of *P. aeruginosa* status:

- *P. aeruginosa* isolated at clinical stability
- Chronic colonisation with *P. aeruginosa*
- New *P. aeruginosa* isolated in the last 4 weeks
- Participants who have never isolated *P. aeruginosa* previously.

9.3 Results

9.3.1 Sputum and urine cytokines on clinical status in bronchiectasis

The baseline characteristics of the 20 participants are summarised in Table 9-2.

Out of 20 participants with sputum available at pulmonary exacerbation, 15 participants had matched samples available for analysis after treatment and 20 at clinical stability. All 6 of the cytokines tested (IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α) were detected 100% in sputum at all three time-points (at the start of exacerbation, after treatment and clinical stability). However, the number of samples detected that fell outside of the standard concentration curves ranged from 95% (IL-17a) to 10% (TNF-α).

Out of 19 participants with urine available at pulmonary exacerbation, 15 participants had matched samples available for analysis after treatment and 17 at clinical stability. The number of cytokines detected in urine ranged from 60 % (IL-17a) to 100% (TNF-α). The number of samples detected that fell outside the standard concentration curves ranged from 100 % (IL-17a) to 13 % (IL-8). The number of sputum and urinary samples detected and those outside of the standard concentration curves are summarised in Table 9-3.

Table 9-2. Baseline characteristics of cytokine cohort (total n=20)

Baseline variable	Start exacerbation	Post exacerbation	Stability
Mean: (SD)			
Absolute FEV_1/ L	1.25 (±0.54)	1.21 (±0.50)	1.20 (±0.50)
FEV_1 % predicted	45.37 (±18.22)	44.99 (±17.04)	44.35 (±18.31)
Peak Flow L/min	213.55 (±106.47)	206.31 (±114.11)	201.60 (±102.52)
Sputum neutrophils cell/g	7.11 (±0.61)	6.78 (±0.72)	7.09 (±0.58)
CFU PIA (log10 CFU/g)	2.08 (±3.04)	2.89 (3.56)	3.50 (±3.35)
CFU Blood agar (log 10 CFU/g)	6.32 (±1.05)	6.62 (±1.11)	7.46 (±0.65)
CFU PIA PCR ((log 10 CFU/g))	2.62 (±2.82)	2.72 (±3.01)	3.34 (±3.24)
Median: (IQR)			
Serum CRP	13.5 (1 to 23)	5.5 (0-11)	
Blood neutrophil count	7.5 (5.14-10.3)	5.45 (4.4-6.93)	

SD; standard deviation, FEV1; forced expiratory volume, L/min; litres per minute, CFU; colony forming units.

Table 9-3. The number of cytokines detected in sputum and urine

	IL-1 β	IL-6	IL-8	IL-17a	IL-23	TNF-α
Sputum						
No detected: N (%)						
At exacerbation (n=20)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)
After treatment (n=15)	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)
Clinical stability (n=20)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)
Out of range: N (%)						
At exacerbation	6 (30)	4 (20)	16 (80)	18 (90)	7 (35)	2 (10)
After treatment	1 (7)	3 (20)	14 (93)	14 (93)	7 (47)	4 (27)
Clinical stability	4 (20)	5 (25)	17 (85)	19 (95)	6 (30)	4 (20)
Urine	IL-1 β	IL-6	IL-8	IL-17a	IL-23	TNF-α
No detected: N (%)						
At exacerbation (n=19)	19 (100)	19 (100)	19 (100)	14 (74)	17 (89)	19 (100)
After treatment (n=15)	14 (93)	15 (100)	15 (100)	9 (60)	13 (87)	15 (100)
Clinical stability (n=17)	16 (94)	16 (94)	16 (94)	12 (71)	14 (82)	17 (100)
Out of range: N (%)						
At exacerbation	16 (84)	12 (63)	5 (26)	14 (100)	11 (65)	17 (89)
After treatment	8 (57)	10 (67)	2 (13)	9 (100)	7 (54)	14 (93)
Clinical stability	14 (88)	8 (50)	3 (18)	11 (92)	10 (71)	16 (94)

No detected; Number of samples that had a cytokine detected (concentrations over zero), Out of range; the number of samples detected but concentrations fell outside of the standard curve, all concentrations measured as pg/ml.

9.3.1.1 Correlations between sputum and urinary cytokines

The correlations of cytokines in sputum and urine are described in Table 9-4. There were no correlations between sputum and urinary cytokines at the beginning of a pulmonary exacerbation or after treatment. At clinical stability, TNF-α had a positive correlation in sputum and urine (r=0.720, *p*=0.001).

Table 9-4. Correlations of cytokines in sputum and urine

Spearman's correlation: r(p-value)	
Cytokine	Sputum and Urine
Exacerbation	
IL-1 β	0.062 (0.802)
IL-6	0.081 (0.742)
IL-8	0.345 (0.148)
IL-17a	-0.115 (0.638)
IL-23	-0.068 (0.783)
TNF-α	-0.219 (0.369)
Post exacerbation	
IL-1 β	0.404 (0.152)
IL-6	0.090 (0.759)
IL-8	0.490 (0.075)
IL-17a	0.399 (0.158)
IL-23	0.054 (0.854)
TNF-α	0.117 (0.691)
Stability	
IL-1 β	0.058 (0.826)
IL-6	0.333 (0.191)
IL-8	0.108 (0.680)
IL-17a	0.078 (0.765)
IL-23	-0.118 (0.652)
TNF-α	**0.720 (0.001)**
IL; interleukin, IL-1b; IL-1 beta, TNF-α;TNF-alpha	

9.3.1.2 Comparing levels of pro-inflammatory cytokines between the beginning of a pulmonary exacerbation with after treatment

In sputum, the median level of TNF-α decreased from 73.1 pg/ml (IQR: 26-127) at the beginning of a pulmonary exacerbation to 28.1 pg/ml (IQR: 6.3-55, *p*=0.020, Table 9-5). There were no other differences in sputum or urine levels of IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α between pulmonary exacerbation and after treatment or clinical stability.

9.3.1.3 Comparing levels of pro-inflammatory cytokines between the beginning of a pulmonary exacerbation and clinical stability

In urine, the median level of IL-23 decreased from 106.1 pg/ml (IQR: 62-160) at the beginning of a pulmonary exacerbation to 86.3 pg/ml (IQR: 58-114, *p*=0.014, Table 9-6) at clinical stability. There were no other differences in sputum or urinary levels IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α between pulmonary exacerbation and clinical stability.

Table 9-5. Median levels and IQR of pro-inflammatory cytokines in sputum and urine between beginning of a pulmonary exacerbation and after treatment.

	Start exacerbation	Post exacerbation	*p*-value
Sputum	N=15	N=15	
IL-1 β	794.4 (220.3- 4927.4)	1106.7 (102.3-2112.4)	0.334
IL-6	35.1 (12.8-239.6)	57.7 (6.5-198.3)	0.691
IL-8	1604.0 (1284.5-2141.7)	1414.6 (1201.8-1820.3)	0.364
IL-17a	4.9 (3.6-7.6)	5.7 (4.5-9.4)	0.292
IL-23	171.6 (65.7-340.6)	209.8 (68.1-337.4)	0.955
TNF-α	**73.1 (26.6-127.2)**	**28.1 (6.3-55.0)**	**0.020**
Urine	N=15	N=15	
IL-1 β	2.6 (2.2-4.5)	3.7 (2.0-24.5)	0.233
IL-6	2.1 (1.0-9.2)	2.4 (1.4-7.8)	0.111
IL-8	9.2 (3.8-22.6)	10.2 (5.7-34.2)	0.496
IL-17a	0.9 (0.0-2.0)	0.6 (0.0-2.6)	0.886
IL-23	106.1 (62.2-159.8)	90.3 (61.9-178.4)	0.221
TNF-α	1.0 (0.6-2.1)	1.3 (0.4-2.4)	0.733

IL; interleukin, IL-1b; IL-1 beta, TNF-α;TNF-alpha, values in bold are considered significant and are calculated by Mann Whitney U tests.

Table 9-6. Median levels and IQR of pro-inflammatory cytokines in sputum and urine between beginning of a pulmonary exacerbation and clinical stability.

	Exacerbation	Clinical Stability	*p*-value
Sputum	N=20	N=20	
IL-1 β	794.4 (220.3- 4927.4)	807.1 (254.4-4065.0)	0.601
IL-6	35.1 (12.8-239.6)	46.4 (7.9-310.0)	0.167
IL-8	1604.0 (1284.5-2141.7)	1345.0 (1163.3-1663.6)	0.126
IL-17a	4.9 (3.6-7.6)	6.1 (3.9-7.7)	0.478
IL-23	171.6 (65.7-340.6)	303.3 (111.8-390.9)	0.191
TNF-α	73.1 (26.6-127.2)	71.6 (23.3-253.2)	0.823
Urine	N=17	N=17	
IL-1 β	2.6 (2.2-4.5)	2.6 (2.1-5.3)	0.758
IL-6	2.1 (1.0-9.2)	3.7 (0.7-7.8)	0.149
IL-8	9.2 (3.8-22.6)	10.3 (4.8-22.1)	0.925
IL-17a	0.9 (0.0-2.0)	0.6 (0.0-0.9)	0.244
IL-23	**106.1 (62.2-159.8)**	**86.3 (57.7-113.9)**	**0.014**
TNF-α	1.0 (0.6-2.1)	0.9 (0.7-1.3)	0.478

IL; interleukin, IL-1b; IL-1 beta, TNF-α;TNF-alpha, values in bold are considered significant and are calculated by Mann Whitney U tests.

9.3.1.4 Pro-inflammatory cytokines and clinical outcomes

Further analyses were performed on urinary levels of TNF-α and IL-23 as these cytokines significantly changed with exacerbation status in this cohort. Associations between urinary TNF-α and IL-23 with lung function (FEV_1/L), sputum and blood neutrophil count and bronchiectasis severity index (BSI) were analysed (summarised in Table 9-7).

Levels of urinary TNF-α (pg/ml) above the median at clinical stability were associated with a higher BSI score; 15.1 (SD±2.2) compared to below the median; 11.0 (SD± 3.2, *p*=0.023). No other correlations were noted between urinary levels of TNF-α and IL-23 with lung function (FEV_1/L), sputum and blood neutrophil count and bronchiectasis severity index (BSI).

Table 9-7. Urine levels of IL-23 and TNF-α and clinical outcomes at the beginning of a pulmonary exacerbation, after treatment and clinical stability.

At Exacerbation	IL-23 ≤	IL-23 >	p-value	TNF-α≤	TNF-α >	p-value
FEV1/L	1.3 (±0.5)	1.3 (±0.5)	0.979	1.3 (±0.5)	1.3 (±0.5)	0.716
Sputum neuts	7.1 (±0.5)	7.1 (±0.7)	0.881	7.0 (±0.7)	7.1 (±0.5)	0.769
Blood neuts*	9.1 (5.6-10.6)	6.4 (4.4-10.1)	0.457	7.9 (5.6-10.0)	7.7 (4.4-10.6)	0.713
After treatment	IL-23 ≤	IL-23 >	p-value	TNF-α≤	TNF-α >	p-value
FEV1/L	1.4 (±0.5)	1.0 (±0.4)	0.095	1.3 (±0.5)	1.1 (±0.5)	0.330
Sputum neuts	6.6 (±0.9)	7.0 (±0.5)	0.327	6.8 (±0.8)	6.7 (±0.7)	0.813
Blood neuts*	5.9 (5.1-7.3)	5.8 (4.7-7.1)	0.862	5.5 (4.4-6.7)	6.1 (5.2-9.0)	0.224
Clinical stability	IL-23 ≤	IL-23 >	p-value	TNF-α≤	TNF-α >	p-value
FEV1/L	1.3 (±0.5)	1.3 (±0.5)	0.738	1.4 (±0.4)	1.1 (±0.5)	0.100
Sputum neuts	7.3 (±0.6)	6.9 (±0.6)	0.168	7.0 (±0.6)	7.1 (±0.7)	0.792
BSI score	13.7 (±3.8)	12.8 (±2.6)	0.574	**11.9 (±3.2)**	**15.1 (±2.2)**	**0.036**

Concentrations are measured as pictogram per millilitre. ±; represents standard deviation, *-represents median and interquartile range in brackets, sputum neutrophils measured as $\log_{10}$ cells per gram, blood neutrophils measured as 10^{9}/L and data were not available at clinical stability, BSI; bronchiectasis severity score, ≤; less than or equal to median concentration, >; greater than median concentration. Values in bold are represented as $p<0.05$ and are determined by Two-sample t-tests or Mann-Whitney U tests for blood neutrophils.

9.3.1.5 Summary of pro-inflammatory cytokines in sputum and urine and clinical status in non-CF bronchiectasis

This is the first study to investigate the correlation of sputum and urinary cytokines in a prospective non-CF bronchiectasis cohort experiencing a pulmonary exacerbation. With the exception of TNF-α levels at clinical stability, there were no robust correlations between sputum and urinary measurements of IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α.

The lack of correlation between sputum and systemic cytokines (measured in blood) has been previously described [466, 467]. Possibilities for this include the variability of sampling techniques such as dilution protocols. In addition, sputum may reflect compartmentalized inflammatory responses in the lung compared to systemic measures [468]. However, it has been postulated that systemic markers of

inflammation may arise due to local 'overflow' from bronchial inflammation and may reflect severe forms of disease [469]. Despite an absence of consistent correlations between sputum and urinary cytokines, we have found that urinary cytokines are measurable in this population which may be clinically useful in future.

From our data, we found TNF-α was the most promising cytokine in this cohort that may correlate with clinical status and disease progression. It was detected in 100% of urine and sputum samples from people with non-CF bronchiectasis. We found sputum levels of TNF-α significantly reduced between presentation with a pulmonary exacerbation and after treatment with antibiotics as has been previously reported [462]. Furthermore, higher urinary levels of TNF-α at clinical stability correlated with a greater bronchiectasis severity score, a finding consistent with Martinez-Garcia *et al,* whereby plasma levels reflected higher severity scores and systemic markers of inflammation [470]. In addition, Guran *et al* reported that high resolution CT scan severity scores correlated positively with sputum TNF-α levels in a cohort of children with non-CF bronchiectasis [471]. However, there have been no previous reports of studies of the urinary levels of TNF-α in CF or bronchiectasis.

Furthermore, we found urinary levels of IL-23 were lower at clinical stability compared to pulmonary exacerbation. The importance of IL-23 in initiating airway inflammation in response to *P. aeruginosa* has been reported by Dubin *et al* [472, 473]. In addition, IL-23 sputum levels have been shown to correlate with active infection in CF and is therefore consistent with our findings [474, 475]. In summary, this is the first time urinary levels of IL-23 have been shown to potentially reflect clinical status in individuals with non-CF bronchiectasis and further study is warranted.

9.3.2 Urinary biomarkers of *P. aeruginosa* status at clinical stability in non-CF bronchiectasis

At clinical stability, 17 urine samples were available for cytokine analysis and 37 were available for urinary lateral flow assay (Mologic ltd) analysis (as described in Chapter 8). The purpose of this study was to investigate the potential of non-invasive urinary biomarkers to reflect *P. aeruginosa* burden of infection in people with non-CF bronchiectasis.

9.3.2.1 Correlation with P. aeruginosa load measured by culture and PCR

9.3.2.1.1 Urinary lateral flow assays

The correlations of the 5 urinary inflammatory markers; NGAL, CC16, TIMP-1, Fibrinogen and CRP with *P. aeruginosa* load detected by culture and PCR are summarised in Table 9-8.

Concentrations of urinary NGAL (ng/ml) were positively correlated with *P. aeruginosa* load measured by culture (r=0.394, *p*=0.042) and PCR (r=0.450, *p*=0.014). In addition, urinary TIMP-1 concentrations (ng/ml) were positively correlated with *P. aeruginosa* measured by culture (r=0.442, *p*=0.021). No other correlations were noted between the urinary inflammatory markers and *P. aeruginosa* load.

9.3.2.1.2 UrinaryTh17 pro-inflammatory cytokines

The correlations of 6 Th17 pro-inflammatory cytokines IL1β, IL-6, IL-8, IL-17a, IL-23 and TNF-α) with *P. aeruginosa* load detected by culture and PCR are summarised in Table 9-8. The concentration of urinary TNF-α (pg/ml) was positively correlated with *P. aeruginosa* load measured by culture (r=0.510, *p*=0.036). No other correlations were present between any of the urinary pro-inflammatory cytokines and *P. aeruginosa* load.

Table 9-8. Correlations of cytokines and urinary inflammatory markers with *P. aeruginosa* load at clinical stability.

	Spearman's Correlation: r(*p*-value)	
	P. aeruginosa Culture	*P. aeruginosa* PCR
Mologic		
NGAL	**0.394 (0.042)**	**0.450 (0.014)**
CC16	-0.044 (0.829)	0.087 (0.652)
TIMP	**0.442 (0.021)**	0.175 (0.365)
Fib	0.079 (0.702)	-0.057 (0.773)
CRP	0.136 (0.499)	-0.127 (0.513)
Cytokine		
IL-1 β	0.177 (0.497)	0.141 (0.590)
IL-6	0.016 (0.952)	-0.032 (0.903)
IL-8	0.356 (0.161)	0.271 (0.293)
IL-17a	0.213 (0.411)	0.298 (0.245)
IL-23	-0.245 (0.344)	-0.064 (0.808)
TNF-α	**0.510 (0.036)**	0.391 (0.121)

9.3.2.2 Urinary cytokines and inflammatory markers on P. aeruginosa status

9.3.2.2.1 *Urinary lateral flow assays*

Urinary levels of NGAL (ng/ml) were higher if *P. aeruginosa* was isolated in sputum at clinical stability; 28.9 (IQR: 16-43) compared to if it was not; 11.1 (IQR: 3-31, *p*=0.042). No other urinary inflammatory markers were associated with *P. aeruginosa* isolation at clinical stability (Table 9-9).

Urinary levels of NGAL (ng/ml) were higher if the participant was chronically colonised with *P. aeruginosa*; 29.5 ng/ml (IQR: 14-53), compared to if they were not; 10.3 ng/ml (IQR:4-27, *p*=0.017). On the contrary, CC16 urinary levels were lower if the participant was chronically colonised with *P. aeruginosa*; 206.4 ng/ml (IQR: 123-607), compared to if they were not; 387.5 (IQR: 288-1469, *p*= 0.036).

In addition, urinary levels of CC16 were higher if *P. aeruginosa* was newly isolated within the last 4 weeks; 1678 ng/ml (IQR: 1648-1882), compared to if it was not; 302 ng/ml (IQR: 147-607, *p*=0.008). No other associations were noted between chronic colonisation and new isolation of *P. aeruginosa*.

In participants who had never isolated *P. aeruginosa* previously, urinary NGAL levels were lower; 4.7 ng/ml (IQR: 2-12) compared to if they had isolated *P. aeruginosa* previously; 25.7 ng/ml (IQR: 11-42, p=0.015). Similarly, urinary levels of TIMP-1 were lower if participants had never isolated *P. aeruginosa*; 0.4 ng/ml (IQR: 0.3-1.5), compared to if they had isolated it previously; 2.0 (IQR: 0.6-2.8, p=0.038).

9.3.2.2.2 Pro-inflammatory cytokines

Levels of urinary TNF-α were higher if *P. aeruginosa* was isolated from sputum at clinical stability; 1.2 pg/ml (IQR: 0.9-3.3), compared to if it was not; 0.7 pg/ml (IQR: 0.6-0.9, p=0.023, Table 9-9). There were no other correlations with any urinary cytokines and *P. aeruginosa* status.

Table 9-9. Investigation of urinary inflammatory markers and urinary cytokines with *P. aeruginosa* status.

Mologic N=37	PsA isolated at stability	PsA not isolated at stability	New PsA in last 4 weeks	No new PsA in last 4 weeks
n (%)	n=14 (48)	15 (52)	n=5 (14)	32 (86)
NGAL	**28.9 (16.4-43.3)**	**11.1 (2.8-30.5)**	24.4 (5.8-24.4)	19.2 (4.7-41.1)
CC16	328.3 (159.1-1211)	317.1 (288-1404)	**1678 (1648-1882)**	**302.0 (144.6-607.0)**
TIMP	2.3 (0.6-2.8)	1.5 (0.5-2.7)	4.3 (1.0-5.3)	1.4 (0.5-2.6)
Fib	8.8 (3.2-26.7)	17.7 (5.1-35.9)	0.4 (0-26.7)	17.7 (5.1-35.9)
CRP	0.1 (0.0-1.5)	0.2 (0.0-1.3)	0.2 (0.2-0.4)	0.2 (0.0-1.3)
	PsA colonised	Not PsA colonised	Has Isolated PsA previously	Never isolated PsA
n (%)	19 (51)	18 (49)	30 (81)	7 (19)
NGAL	**29.5 (13.5-52.9)**	**10.3 (3.6-26.9)**	**25.7 (11.1-41.5)**	**4.7 (1.6-11.7)**
CC16	**206.4 (122.8-607)**	**387.5 (288-1469)**	317.1 (159.1-1211)	288 (125.4-1404)
TIMP	1.2 (0.5-2.6)	1.7 (0.4-4.3)	**2.0 (0.6-2.8)**	**0.4 (0.3-1.5)**
Fib	17.7 (3.2-61.6)	17.7 (0.4-32.2)	19.5 (3.2-35.9)	12.4 (5.1-23.1)
CRP	0.2 (0.0-1.3)	0.2 (0.0-1.1)	0.3 (0.0-1.2)	0.2 (0-1.3)

PsA; *Pseudomonas aeruginosa*, N; number of participants with data available, n; number of participants, values in bold are significant at p<0.05 determined by Mann-Whitney U test.

Cytokines N=17	PsA isolated at stability	PsA not isolated at stability	New PsA in last 4 weeks	No new PsA in last 4 weeks
n (%)	8 (47)	9 (53)	2 (12)	15 (88)
IL-1 β	3.0 (1.4-12.1)	2.5 (2.4-4.1)	4.4 (3.4-5.3)	2.5 (2.1-6.5)
IL-6	4.4 (0.5-14.6)	3.7 (1.3-7.8)	6.5 (5.3-7.8)	3.5 (0.6-8.3)
IL-8	15.8 (6.6-35.0)	6.5 (1.7-18.2)	14.3 (6.5-22.1)	10.3 (2.4-27.4)
IL-17a	0.8 (0.3-2.0)	0.6 (0-0.9)	1.5 (0.9-2.0)	0.6 (0-0.9)
IL-23	71.8 (51.4-111.8)	98.2 (74.2-113.9)	88.3 (86.3-90.3)	77.6 (45.1-133.2)
TNF-α	**1.2 (0.9-3.3)**	**0.7 (0.6-0.9)**	0.8 (0.7-0.9)	0.9 (0.7-1.4)
	PsA colonised	Not PsA colonised	Has Isolated PsA previously	Never isolated PsA
n (%)	8 (47)	9 (53)	14 (82)	3 (18)
IL-1 β	2.3 (0.7-11.2)	3.4 (2.5-5.3)	4.4 (3.4-5.3)	2.5 (2.1-6.5)
IL-6	2.1 (0.5-14.6)	5.3 (2.4-7.8)	6.5 (5.3-7.8)	3.5 (0.6-8.3)
IL-8	9.7 (3.6-34.2)	10.3 (6.2-22.1)	14.3 (6.5-22.1)	10.3 (2.4-27.4)
IL-17a	0.6 (0.1-1.5)	0.9 (0-0.9)	1.5 (0.9-2.0)	0.6 (0-0.9)
IL-23	61.9 (22.5-105.4)	98.2 (86.3-113.9)	88.3 (86.3-90.3)	77.6 (45.1-133.2)
TNF-α	1.2 (0.8-3.3)	0.9 (0.7-0.9)	0.8 (0.7-0.9)	0.9 (0.7-1.4)

PsA; *Pseudomonas aeruginosa*, N; number of participants with data available, n; number of participants, values in bold are significant at p<0.05 determined by Mann-Whitney U tests.

9.3.2.3 Summary of urinary biomarkers and P. aeruginosa status in non-CF bronchiectasis

This is the first study to explore the relationship between urinary cytokines and novel urinary inflammatory markers with *P. aeruginosa* burden in non-CF bronchiectasis.

We showed that urinary NGAL positively correlates with quantitative *P. aeruginosa* load in sputum and isolation at clinical stability. In addition, higher levels of NGAL are present in participants who are chronically colonised or have isolated *P. aeruginosa* previously. In contrast, CC16 levels are lower when a participant is chronically colonised and higher if new isolation of *P. aeruginosa* has occurred and TNF-α levels are higher if *P. aeruginosa* is isolated from sputum at clinical stability.

It is well known that chronic colonisation of *P. aeruginosa* leads to progressive lung damage and frequent pulmonary exacerbations [476]. Chronic colonisation in bronchiectasis is defined by the isolation of potentially pathogenic bacteria in sputum culture on two or more occasions, at least 3 months apart in a 1-year period [6, 365]. Therefore, this definition is reliant on diagnostic criteria which must include adequate sputum specimens being sent for microbiological surveillance at an appropriate frequency. In addition, it relies on reviewing historical clinical records which may be prone to inaccuracy. Serological tests may be useful for defining chronic infection with *P. aeruginosa* but cannot be used to detect early colonisation [477-479] .

NGAL (neutrophil gelatinase-associated lipocalin) has 2 main functions; it acts as an anti-bacterial host defence protein and as a physiological iron barrier [480]. It is well known that in order to survive, bacteria need iron as a cofactor for many metabolic enzymes and therefore produce iron-chelating molecules known as siderophores [481]. *P. aeruginosa* produces two siderophores; pyoverdine and pyochelin, both of which are important in biofilm development and bacterial virulence [482]. NGAL is a molecule that specifically targets bacterial siderophores to reduce virulence, and NGAL has been shown to be elevated in serum in people with CF who are colonised with *P. aeruginosa* compared to those who are not [483]. In addition, It has been

previously shown that peripheral monocytes from subjects with CF secreted NGAL upon infection with *P. aeruginosa* [480]. Interestingly, Peek *et al* discovered that pyoverdine evades NGAL recognition therefore potentially allowing *P. aeruginosa* to establish infections and persist in the lungs in people with CF [481]. Nevertheless, as NGAL functions as a siderophore inhibitor, the positive correlation in our data of urinary levels of NGAL with *P. aeruginosa* burden, presence and chronic colonisation in people with non-CF bronchiectasis suggests further investigation is warranted.

The respiratory lung epithelium protects against environmental insult by maintaining tight junctions between cells, producing detoxifying enzymes and generating the anti-inflammatory club secretory protein; CC16 [484]. In Chapter 8, we described the potential CC16 protective abilities by demonstrating urinary levels of CC16 were raised after treatment for a pulmonary exacerbation compared to the start of an exacerbation. In addition, we reported higher levels of CC16 were associated with a longer time to next pulmonary exacerbation. Hayashida *et al* showed that acute pulmonary infection with *P. aeruginosa* inhibited Clara cell secretory protein (also called CC16) in mice and CC16 deficiency was associated with increased influx of polymorphonuclear cells and improved killing of *P. aeruginosa* in vivo [485]. However, CC16 subsequently inhibited neutrophil recruitment and ongoing inflammation after lung infection with *P. aeruginosa* [485]. Furthermore, another study found that in CC16- deficient mice, chronic *P. aeruginosa* inflammation resulted in chronic bronchitis and emphysematous changes [486]. Therefore, CC16 plays an important role in modulating inflammation after acute pulmonary infections and in protecting the host from chronic inflammation induced lung damage. Our data is consistent with these findings as we showed urinary levels of CC16 were reduced when people were colonised with *P. aeruginosa* and were conversely higher in people with new isolation of *P. aeruginosa*.

We also showed that urinary levels of TNF-α were higher in people who isolated *P. aeruginosa*. This finding is biologically plausible as Cusomano *et al* demonstrated the ability of *P. aeruginosa* to induce the release of TNF-α from human leukocytes [487].

Interestingly, Martinez-Garcia *et al* found elevated plasma TNF-α concentrations were associated with extent of non-CF bronchiectasis, respiratory failure and chronic colonization of *P. aeruginosa* [470].

In summary, this study has showed for the first time that urinary inflammatory markers and urinary pro-inflammatory cytokines are associated with early or chronic colonisation with *P. aeruginosa*. These findings are in keeping with the pro inflammatory response associated with *P. aeruginosa* infection and warrant further investigation to determine if elevated urinary cytokines could provide an early indication of new infection.

9.4 Discussion

This chapter describes two novel studies; i) the investigation of urinary pro-inflammatory cytokines and clinical status in people with bronchiectasis and, ii) the exploration of urinary markers of inflammation with *P. aeruginosa* burden.

Although clinicians use a range of criteria including clinical assessments, severity scores and lung function parameters in people with bronchiectasis, there are currently no validated biomarkers used to assess clinical status, severity or prognosis in bronchiectasis.

9.4.1 Strengths

Strengths of this study includes the use of a prospective observational cohort of people with bronchiectasis experiencing a pulmonary exacerbation that have also been assessed after treatment and at clinical stability. In addition, despite sputum and plasma pro-inflammatory cytokines being investigated in this population, there are currently no recorded studies exploring urinary cytokines.

9.4.2 Limitations

There are several limitations that must be considered when interpreting these data. Firstly, this was a small cohort of 20 people recruited from one centre. Secondly, the

multiplex immunoassay system used to analyse the cytokines had not been validated for urinary samples. Therefore, if further investigation into the role of urinary cytokines in this patient population is considered, a spike/recovery test will be necessary to ensure the test is appropriately validated for the correct sample type. In addition, there were many sputum and urinary cytokines that were detected but fell outside the standard curve of concentrations. Therefore, for these measurements, the precise concentration of the analytes could not be accurately determined and the potential conclusions drawn are subsequently limited. This may also mean that the multiplex assay was not sufficiently sensitive to measure the concentrations of the analytes present in the samples. Further more sensitive platforms should be explored for future studies with pro-inflammatory cytokines in this population. Finally, these data were not corrected for multiple testing and should be regarded as hypothesis-generating with scope to investigate these preliminary findings in future studies.

9.4.3 Summary

Despite these limitations, we have shown that urinary cytokines can be detected in people with non-CF bronchiectasis and correlate with severity scores as well as clinical status in these individuals. In addition, urinary cytokines and novel urinary inflammatory markers correlate with *P. aeruginosa* burden in this population and may have potential as non-invasive biomarker of *P. aeruginosa* colonisation status.

Chapter 10. Conclusions and future work

10.1 Summary of key findings

This thesis investigated the microbiological and clinical factors involved in disease progression, clinical status and airway infection with *P. aeruginosa* in people with CF and non-CF bronchiectasis.

10.1.1 Long-term prospective follow-up in adults with CF

We describe an association of the C9-PQS molecule present in sputum and increased antibiotic usage for pulmonary exacerbations in adults with CF over an 8-year follow up period. There remains much to be learned about AQ production and regulation in disease progression in CF and further prospective studies are needed. However, the development of anti-virulence drugs or antibodies that target the AQ-dependant QS pathway is a promising future consideration in this cohort [221].

Using a novel live/dead separation technique (PMA), we found two of the most abundant anaerobic species present in CF sputum were associated with long-term lung function decline in a cohort predominately colonised with *P. aeruginosa*. Currently, evidence is conflicted regarding the pathological or protective role of anaerobic bacteria in CF. It is possible that some anaerobic species are more pathological in the presence of *P. aeruginosa*, or through microbial interactions with anaerobic bacteria, the virulence of *P. aeruginosa* is increased in the CF lung environment [343, 357].

10.1.2 Clinical and microbiological factors influencing disease progression in bronchiectasis

There is an ongoing need for more research in non-CF bronchiectasis and to understand the many factors involved in pulmonary exacerbations, disease severity and mortality. Fifty participants experiencing a pulmonary exacerbation and requiring IV antibiotics were recruited into an observational cohort study. The baseline

demographics of participants were comparable to other large cohort studies; however, our study cohort had a higher exacerbation frequency and disease severity.

We found the levels of systemic inflammatory markers such as CRP, blood neutrophil count and sputum neutrophil count reduced after treatment for a pulmonary exacerbation and correlated with clinical improvement. In addition, after treatment for a pulmonary exacerbation, the sputum and blood neutrophil count correlated negatively with lung function. It is important to note that the levels of CRP and blood neutrophils at the start of treatment were not markedly raised and it was in fact the relative reduction of serum CRP and blood neutrophils after treatment that correlated with lung function improvement. For example, in our cohort, the median CRP reduced from 13 at the beginning of exacerbation to 5 after treatment. This has been noted in previous studies whereby higher levels of systemic inflammation correlate with severity of an exacerbation but a normal CRP does not exclude a pulmonary exacerbation warranting treatment [488]. Our data is consistent with previous studies whereby spirometric measures of lung function cannot reliably determine an exacerbation or treatment response [375]. In fact, the most consistent clinical measurement that correlated with clinical status was the quality of life score, which has been previously described [374].

We found the *P. aeruginosa* quantitative load measured by culture did not reduce between the beginning of an exacerbation and after treatment, but when measured by qPCR using the PMA method, the quantitative load reduced. This finding was also reflected in our CF cohort in Chapter 3, whereby the same discrepancy in the quantification of *P. aeruginosa* load measured by culture and PCR was noted. We conclude that our observations show qPCR using PMA is potentially a more reliable method for accurately quantifying bacterial load compared to culture. The amount of *P. aeruginosa* (culture and qPCR) quantified after treatment for an exacerbation negatively correlated with lung function. Interestingly, those participants colonised with *P. aeruginosa* had a greater decline in lung function (measured as peak flow) after IV antibiotics compared to those who were not.

Finally, we describe older age, lower lung function and current smokers were all associated with a higher exacerbation risk and mortality. In our cohort, male gender was associated with exacerbation risk. In addition, a higher blood neutrophil count at the end of treatment was associated with a 12% increased risk of exacerbation. A novel finding in our cohort showed chronic colonisation with non-mucoid strains of *P. aeruginosa* was associated with a higher exacerbation risk, which is contrary to data in CF [398, 399]. Similarly, a shorter time colonised with *P. aeruginosa* was associated with increased mortality. These factors may be explained by a reduction in virulence factor production due to long-term colonisation, however, this has not been previously recognised in non-CF bronchiectasis.

10.1.3 Urinary biomarkers of clinical status in CF and bronchiectasis

We investigated five novel urinary inflammatory molecules (NGAL, CC16, TIMP-1, Fibrinogen and CRP) in people with CF and non-CF bronchiectasis and explored if these correlated with clinical status. In summary, we demonstrated urinary CC16 was significantly higher between the start and end of treatment for pulmonary exacerbations, significantly lower at clinical stability in CF and non-CF bronchiectasis patients. Urinary CRP levels were lower after treatment for a pulmonary exacerbation in CF and lower at clinical stability compared to a pulmonary exacerbation in bronchiectasis. In addition, lower urinary CC16 levels and higher urinary TIMP-1 levels were associated with future risk of a pulmonary exacerbation in non-CF bronchiectasis.

We also reported two promising urinary pro-inflammatory cytokines; IL-23 and TNF-α in non-CF bronchiectasis patients. We showed higher levels of TNF-α in the urine correlated with sputum levels and were positively associated with a higher severity score in bronchiectasis. We also demonstrated urinary IL-23 levels were lower at clinical stability compared to the beginning of a pulmonary exacerbation. Although further studies are needed to investigate non-invasive urinary biomarkers, we report for the first time their potential in demonstrating clinical status in both CF and non-CF bronchiectasis.

10.1.4 Biomarkers of *P. aeruginosa*

A minimally-invasive diagnostic test for *P. aeruginosa* would be especially useful in young children with CF and may therefore avoid need for more invasive tests such as bronchoalveolar lavage. In addition, surrogate systemic measures of *P. aeruginosa* burden may prove increasingly useful in the era of highly effective CTFR modulator therapy where spontaneous sputum production is likely to decrease in the longer term.

We explored whether three previously defined AQs; HHQ NHQ, and HQNO, correlated more strongly with culture-independent measures of live *P. aeruginosa* load compared to a culture-dependent method. Overall, we found stronger correlations between AQ concentrations measured in blood, urine and sputum with qPCR measures of bacterial load compared to culture. In addition, absolute change of sputum *P. aeruginosa* load measured by qPCR were reflected by an absolute change in plasma NHQ between the start and end of IV antibiotics for pulmonary exacerbation.

For the first time, we investigated if AQs were detectable in salivary samples in adults with CF with known *P. aeruginosa* pulmonary infection. All six AQs (HHQ, HQNO, NHQ, NQNO, C7-PQS and C9-PQS) were detected in salivary samples and there were positive correlations between concentrations of AQs measured in saliva and corresponding sputum samples. In addition, salivary concentrations of four AQs (HHQ, NHQ, HQNO and NQNO) correlated significantly with quantitative load of sputum *P. aeruginosa* measured using PCR.

Finally, we investigated if urinary inflammatory molecules and pro-inflammatory cytokines correlated with sputum *P. aeruginosa* load measured using PCR and *P. aeruginosa* status in people with bronchiectasis. We showed that urinary NGAL levels positively correlated with quantitative *P. aeruginosa* load in sputum and isolation at clinical stability. In addition, higher levels of urinary NGAL were present in participants who were chronically colonised or had isolated *P. aeruginosa* previously. In contrast,

urinary CC16 levels were lower when a participant was chronically colonised with *P. aeruginosa* and higher if new isolation of *P. aeruginosa* had occurred. Urinary TNF-α levels were higher if *P. aeruginosa* was isolated from sputum at clinical stability. In summary, we demonstrated for the first time that novel urinary inflammatory markers and urinary pro-inflammatory cytokines may predict early or chronic colonisation with *P. aeruginosa* in people with non-CF bronchiectasis.

10.2 Future areas of research

As part of the work reported in this thesis, we have established a cohort of 50 participants with bronchiectasis. This will enable us to explore future areas of research and permit long-term follow up in this cohort.

10.2.1 Exploring quorum sensing molecules in bronchiectasis

We have shown both in this thesis and previous studies that AQ quorum sensing molecules are associated with *Pseudomonas aeruginosa* load, increase at pulmonary exacerbation and subsequently reduce after intravenous antibiotic treatment in adults with CF [279, 280]. In our bronchiectasis cohort of 50 participants, sputum, blood and urine samples were sent for QS analysis at *The Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham, UK.*

We aimed to investigate; i) if AQ molecules can be detected in sputum, blood and urinary in a bronchiectasis cohort, ii) to assess AQ levels and their association with clinical status in bronchiectasis, iii) to determine the effect of antibiotics for a pulmonary exacerbation on AQ levels, iv) to explore to potential of AQ molecules as biological markers for *P. aeruginosa* infection.

10.2.2 Whole genome sequencing of micro-organisms in bronchiectasis

In our non-CF bronchiectasis cohort, we obtained sputum samples and performed quantitative microbiological culture using blood agar and Pseudomonas isolation agar. In addition, we beaded and stored the individual organisms grown on these

media. By doing this, we aim to sequence the genomes of individual isolates of *P. aeruginosa* in non-CF bronchiectasis. From this, we aim to determine whether each individual with non-CF bronchiectasis carries the same or different line of this pathogen and by *utilising in-silico* analysis, whether the same line of this pathogen has been detected elsewhere [489].

We hypothesise that the same line of this pathogen is carried by a proportion of the bronchiectasis population. If so, this raises important clinical questions such as whether this shared line is associated with more pathophysiological damage and longer term adverse clinical outcomes. If so, there may be parallels with the epidemic spread of this pathogen in other infections including CF.

In addition, we stored the mixed cultures of species isolated on blood agar. By phenotypic screening and use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF), we can determine which species – primarily commensal in nature – are present [490]. Once we have identified pairs of isolates that are present both at pulmonary exacerbation and at clinical stability, we aim to fully sequence the genome of the commensal or pathogenic species. By doing this, we can compare pairs of the same species from the same individual in longitudinal samples and explore differences in the genes expressed at different time points (at the start and end of a pulmonary exacerbation and clinical stability).

Sequence data will also serve to assess whether lines of commensal species are shared by individuals with non-CF bronchiectasis at various disease states such as at pulmonary exacerbation or clinical stability.

10.2.3 The microbiota in bronchiectasis using PMA separation

In collaboration with Dr Kenneth Bruce, sputum samples in our non-CF bronchiectasis cohort were sent to *The Institute of Pharmaceutical Science, King's College London, London, UK*. Using the novel PMA cell separation technique, we aim to characterise the airway microbiota in individuals with non-CF bronchiectasis. The means of analysis will be combining both 16S rRNA gene sequencing and metagenomics

sequencing. This is culture-independent, well-validated and identifies to at least genus level the taxa that are in a sample [491]. Through this analysis, we will be able to correlate the presence of individual taxa with clinical parameters in this cohort. This will be incorporated with lung function decline, mortality, time to next exacerbation, sputum differential cell counts and bacterial load.

10.2.4 Exploring PMA diversity in CF and bronchiectasis

As summarised in Chapter 3 and 5 in this thesis, sputum samples from adults with CF were sent to *The Institute of Pharmaceutical Science, King's College London, London, UK*, for qPCR and microbiome analysis using the PMA separation technique. The use of PMA in CF has been shown to give a more accurate account of viable micro-organisms present in the CF microbiota [492]. PMA-treatment is able to better highlight changes in relative abundance of OTUs in CF sputum whilst not over-estimating the abundance of each viable organism [493]. Using this methodology, we aim to compare the microbiota of individuals with CF and non-CF bronchiectasis at pulmonary exacerbation and clinical stability. In addition, we aim to investigate the impact PMA has on microbiota diversity and relative abundance in comparing the viable/non-viable cells present in both cohort of patients.

10.2.5 Additional areas of research from our findings

From our findings, further prospective investigations are needed in both CF and bronchiectasis cohorts. Furthermore, in the investigation of biomarkers of clinical status and *P. aeruginosa* load, regular longitudinal profiling of sputum, blood, urine and saliva samples are required to determine expected levels of inter-individual variability. In addition, results including AQ measurements in saliva and urine need to be validated in control populations of healthy volunteers and people with CF who do not have pulmonary *P. aeruginosa*. Finally, refinement of the liquid chromatography-tandem mass spectrometry technology is needed to increase the sensitivity of detecting AQs in samples such as saliva. Saliva is particularly interesting as it may

provide the ability to develop non-invasive home monitoring lateral flow tests in the CF population.

10.3 Concluding remarks

Overall, this thesis highlights the investigation of several factors that may influence or predict disease progression and clinical status in CF and bronchiectasis. In an observational CF cohort, we explored long-term outcomes with AQ *P. aeruginosa* quorum sensing molecules and the most abundant anaerobic microbiota present in sputum. We recruited and established a cohort of people with bronchiectasis, which permitted the analysis of key microbiological and clinical factors that may influence disease progression, mortality and pulmonary exacerbations. In addition, we investigated novel urinary inflammatory molecules in CF and bronchiectasis to explore correlations with clinical status. Lastly, we explored novel non-invasive biomarkers of *P. aeruginosa* burden such as salivary and urinary AQs, urinary inflammatory molecules and urinary pro-inflammatory cytokines.

In conclusion, the utilisation of non-invasive biomarkers may pave the way to improve the diagnosis, management and prevention of pulmonary exacerbations in these cohorts. Furthermore, point-of-care biomarkers for pathogens such as *P. aeruginosa* may facilitate earlier recognition of infection and eradication attempts. In future, we have the capacity to utilise these established study cohorts to improve our understanding of the pathophysiology of airway infection and disease progression in both CF and non-CF bronchiectasis.

Appendices

Appendix 1: Health Research Approval

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Dr Helen Barr
Consultant in Respiratory Medicine
Nottingham City Hospital
Respiratory Medicine/CF Centre
Nottingham City Hospital, Hucknall Rd
Nottingham
NG5 4AE

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

17 May 2018

Dear Dr Barr

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Airway infection in bronchiectasis patients: A feasibility study**
IRAS project ID:	**235786**
Protocol number:	**17RM028**
REC reference:	**18/WM/0125**
Sponsor	**Research & Innovation, Nottingham University Hospitals NHS Trust**

I am pleased to confirm that HRA and Health and Care Research Wales (HCRW) Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales?
You should now provide a copy of this letter to all participating NHS organisations in England and Wales*, as well as any documentation that has been updated as a result of the assessment.

*'In flight studies' which have already started an SSI (Site Specific Information) application for NHS organisations in Wales will continue to use this route. Until 10 June 2018, applications on either documentation will be accepted in Wales, but after this date all local information packs should be shared with NHS organisations in Wales using the Statement of Activities/Schedule of Events for non-commercial studies and template agreement/ Industry costing template for commercial studies.

This is a single site study sponsored by the site. The sponsor R&D office will confirm to you when the study can start following issue of HRA and HCRW Approval.

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) has been sent to the coordinating centre of each participating nation. You should work with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?
The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

I am a participating NHS organisation in England or Wales. What should I do once I receive this letter?
You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

Name: Maria Koufali
Tel: 0115 924 9924 ext 70673
Email: researchsponsor@nuh.nhs.uk

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **235786**. Please quote this on all correspondence.

IRAS project ID	235786

Yours sincerely

Chris Kitchen
Assessor

Email: hra.approval@nhs.net

Copy to: *Ms Maria Koufali, Research & Innovation Nottingham University Hospitals NHS Trust (Sponsor and R&D Contact)*

IRAS project ID	235786

List of Documents

The final document set assessed and approved by HRA and HCRW Approval is listed below.

Document	*Version*	*Date*
GP/consultant information sheets or letters [GP letter]		
IRAS Application Form [IRAS_Form_19032018]		19 March 2018
IRAS Checklist XML [Checklist_10052018]		10 May 2018
Participant consent form [Consent form]	1.1	03 May 2018
Participant consent form [Consent form]	1.0	13 April 2018
Participant consent form		
Participant information sheet (PIS) [PIS]	1.0	13 April 2018
Participant information sheet (PIS) [PIS]	1.1	03 May 2018
Research protocol or project proposal [Protocol]	2.0	28 February 2018
Summary CV for Chief Investigator (CI) [CV]		05 March 2018
Summary CV for student [CV]		
Validated questionnaire [QoI B]		

IRAS project ID	235786

Summary of assessment

The following information provides assurance to you, the sponsor and the NHS in England and Wales that the study, as assessed for HRA and HCRW Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England and Wales to assist in assessing, arranging and confirming capacity and capability.

Assessment criteria

Section	Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	The applicant has confirmed that the researcher will be considered part of the care team at site.
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	This is a non-commercial single site study taking place in the NHS where that single NHS organisation is also the study sponsor. Therefore no study agreements are expected.
4.2	Insurance/indemnity arrangements assessed	Yes	No comments
4.3	Financial arrangements assessed	Yes	The study is funded by a grant from the NIHR.
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	IRAS A36 states that personal data will be stored on university computers. The applicant has confirmed that all identifiable data will remain on NHS computers.
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	The Human Tissue Act applies.

IRAS project ID	235786

Section	Assessment Criteria	Compliant with Standards	Comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	No comments
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England and Wales

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.

This is a non-commercial single site study taking place in the NHS where that single NHS organisation is also the study sponsor. If this study is subsequently extended to other NHS organisation(s) in England or Wales, an amendment should be submitted, with a Statement of Activities and Schedule of Events for the newly participating NHS organisation(s) in England or Wales.

The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England and Wales in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. Where applicable, the local LCRN contact should also be copied into this correspondence.

If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England and Wales which are not provided in IRAS, the HRA or HCRW websites, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net or HCRW at Research-permissions@wales.nhs.uk. We will work with these organisations to achieve a consistent approach to information provision.

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and Wales, and the minimum expectations for education, training and experience that PIs should meet (where applicable).

A Principal Investigator is expected to be in place at the participating organization.

GCP training is not a generic training expectation, in line with the HRA/HCRW/MHRA statement on

training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
For research team members that do not have existing contractual relationships with the participating organisation, Honorary Research Contracts should be in place if the activities undertaken at the NHS site involve contact with patients (e.g. to take consent), on the basis of Research passports (if University employed) or NHS to NHS confirmation of pre-engagement checks letters (if NHS employed). The pre-engagement checks should include enhanced DBS checks and Occupational Health Clearance. No specific pre-engagement checks are required to have taken place if the members of the research team are only accessing patients' data.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England and Wales to aid study set-up.
The applicant has indicated that they do not intend to apply for inclusion on the NIHR CRN Portfolio.

Appendix 2: Research Ethics Committee approval

NHS
Health Research Authority

West Midlands - Coventry & Warwickshire Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is an acknowledgement letter from the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

11 May 2018

Dr Karmel Webb
Clinical Research Fellow
University of Nottingham and Nottingham University Hospitals NHS trust
Clinical Science Building
Nottingham City Hospital
Hucknall Rd, Nottingham
NG5 1PB

Dear Dr Webb

Study title:	**Airway infection in bronchiectasis patients: A feasibility study**
REC reference:	**18/WM/0125**
Protocol number:	**17RM028**
IRAS project ID:	**235786**

Thank you for your letter of 10/05/2018. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 03 May 2018

Documents received

The documents received were as follows:

Document	*Version*	*Date*
GP/consultant information sheets or letters [GP letter]		
IRAS Checklist XML [Checklist_10052018]		10 May 2018
Participant consent form [Consent form]	1.1	03 May 2018
Participant consent form [Consent form]	1.0	13 April 2018

Participant information sheet (PIS) [PIS]	1.1	03 May 2018
Participant information sheet (PIS) [PIS]	1.0	13 April 2018
Research protocol or project proposal [Protocol]	2.0	28 February 2018
Summary CV for Chief Investigator (CI) [CV]		05 March 2018
Summary CV for student [CV]		
Validated questionnaire [QoI B]		

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
GP/consultant information sheets or letters [GP letter]		
IRAS Application Form [IRAS_Form_19032018]		19 March 2018
IRAS Checklist XML [Checklist_10052018]		10 May 2018
Participant consent form		
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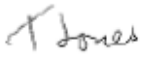
Research protocol or project proposal [Protocol]	2.0	28 February 2018
Summary CV for Chief Investigator (CI) [CV]		05 March 2018
Summary CV for student [CV]		
Validated questionnaire [QoI B]		

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

18/WM/0125 **Please quote this number on all correspondence**

Yours sincerely



Tadeusz Jones
REC Manager

E-mail: NRESCommittee.WestMidlands-CoventryandWarwick@nhs.net

Copy to: *Dr Karmel Webb, University of Nottingham and Nottingham University Hospitals NHS trust*
Ms Maria Koufali, Research & Innovation Nottingham University Hospitals NHS Trust

Appendix 3: Patient information sheet

IRAS ID: 235786

Participant Information Sheet

Version: 1.2 Date: 23.05.2018

Airway infection in bronchiectasis patients

Principle Investigator: Dr Helen Barr

PART 1

1. Invitation

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.

PART 1 tells you the purpose of this study and what will happen to you if you take part.

PART 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

2. What is the purpose of the study?

We aim to see which bugs cause infection in patients with bronchiectasis using a combination of old and new diagnostic tests.

3. Why have I been invited?

You have been invited to participate as we are aware that you are have a diagnosis of bronchiectasis and so may be suitable.

4. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form to confirm that you understand what is involved when taking part in this study. If you decide to take part you are free to leave the study at any time and without giving a reason. If you withdraw, unless you object, we will still keep records relating to the treatment given to you, as this is valuable to the study. A decision to withdraw at any time, or a decision not to take part, will not affect the quality of care you receive.

5. What will happen to me if I take part?

The tests
If you decide to take part we will ask you to provide the following measurements:

1. Blood tests – we will take blood to look for measures of infection. We will store the rest in the freezer. This may be used in the future for studies of substances in the blood which influence bronchiectasis including the genes of any bugs.
2. Sputum production (optional) – this may be used to look for measures of infection and inflammation. This may be used in the future for studies of substances in the sputum which influence bronchiectasis.
3. Blowing test (optional) – this looks at how healthy your lungs are before and after antibiotic treatment.
4. Urine tests- (optional) We will take urine tests to look for measures of infection. This may be used in the future for studies in the urine that influence bronchiectasis.

We will ask you to repeat these measures after treatment with antibiotics and after a period of time when you are not on any antibiotics as this will be useful to see if they have improved.

The samples taken will be processed and we will see if we are able to detect infection molecules in the sputum, blood and urine samples and if this is related to how you are.

We will also use the routine medical data that you provide at regular clinical visits to monitor your health in the future. We may use your medical records to see how your health has been in the past. We will also monitor your health using your hospital and primary care records.

6 What do I have to do?

Participants will be asked to complete a quality of life questionnaire before and after treatment. Participants will complete blowing tests and have blood samples taken at the start and end of antibiotic therapy. In addition, participants will be asked to do a quality of life questionnaire and send further sputum, blood and urine samples when stable during the study period, this is approximately 4-6 week after treatment for a chest infection.
Travel costs for participants will be supplemented by the research team.

7. What is the treatment that is being tested?

We are assessing new methods for detecting bugs in the airways of people with bronchiectasis. Your normal treatments will be continued by your doctor.

8. What are the alternatives for diagnosis or treatment?

Routine sputum samples will be sent to the laboratory and your doctor will continue with treatment as planned.

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

If you do decide to take part in the study, you must report any problems you have to your study nurse or doctor. There is also a contact number given at the end of this information sheet for you

to phone if you become worried at any time. In the unlikely event of an emergency occurring during the conduct of the study, we may contact your nominated next of kin.

10. What are other possible disadvantages and risks of taking part?

None that we are aware of. If you have private medical insurance you are asked to check with the company before taking part in the study.

11. What are the possible benefits of taking part?

We cannot promise the study will help you but the information we get might help improve the treatment of people with bronchiectasis in future.

12. What happens when the research study stops?

When the research stops, the results with be analysed and written up as a publication.

13. What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital or you can contact PALS (Patient Advice and Liaison Service) telephone 0800 183 0204

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

14. Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. Details are included in Part 2.

15. Contact Details

Doctor

Name Dr Karmel Webb Tel. Number: 0115 8231753

Research/Specialist Nurse

Name Jessica Kearsley Tel. Number: 0115 9691169

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART 2

16. What if new information becomes available?

Sometimes during the course of a clinical trial, new information becomes available on the drugs that are being studied. If this happens, we will tell you about it and discuss with you whether you want to or should continue in the study. If you decide to withdraw, we will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

On receiving new information, we might consider it to be in your best interests to withdraw you from the study. If so, we will explain the reasons and arrange for your care to continue.

If the study is stopped for any other reason, you will be told why and your routine care will continue with your doctor.

17. What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. Information collected may still be used. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

18. Will my part in this study be kept confidential?

If you consent to take part in this study, the records obtained while you are in this study as well as related health records will remain strictly confidential at all times. The information will be held securely on paper and electronically at your treating hospital Nottingham University Hospitals NHS Trust under the provisions of the 1998 Data Protection Act. Your name will not be passed to anyone else outside the research team or the sponsor, who is not involved in the trial. You will be allocated a trial number, which will be used as a code to identify you on all trial forms.

If you withdraw consent from further study treatment, unless you object, your data and samples will remain on file and will be included in the final study analysis.

In line with Good Clinical Practice guidelines, at the end of the study, your data will be securely archived for a minimum of 5 years. Arrangements for confidential destruction will then be made.

With your permission, your GP, and other doctors who may be treating you, will be notified that you are taking part in this study.

The information collected about you may also be shown to authorised people from the UK Regulatory Authority and Independent Ethics Committee; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant.

If you withdraw consent from further study treatment, unless you object, your data and samples will remain on file and will be included in the final study analysis.

In line with Good Clinical Practice guidelines, at the end of the study, your data will be securely archived for a minimum of 15 years. Arrangements for confidential destruction will then be made.

With your permission, your GP, and other doctors who may be treating you, will be notified that you are taking part in this study.

Data collected during the study may be transferred for the purpose of (processing, analysis, etc) to associated researchers within/outside the European Economic Area. Some countries outside Europe may not have laws which protect your privacy to the same extent as the Data Protection Act in the UK or European Law. The Sponsor of the trial will take all reasonable steps to protect your privacy. There will be no personal identifiable data that will be transferred.

19. Use of Your Personal Data in Research

Nottingham University Hospitals NHS Trust is the sponsor for this study based in the United Kingdom. We will be using information from you and/or your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.
You can find out more about how we use your information www.nuh.nhs.uk.

Nottingham University Hospitals (NUH) will use your name, NHS number and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from NUH NHS trust and regulatory organisations may look at your medical and research records to check the accuracy of the research study. NUH will pass these details to the NUH sponsor team along with the information collected from you and/or your medical records. The only people in NUH sponsor team who will have access to information that identifies you will be people who need to contact you to send travel reimbursement or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details.
NUH will keep identifiable information about you from this study for 5 years after the study has finished.

When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other

organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research.
This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

Data collected during the study may be transferred for the purpose of (processing, analysis, etc) to associated researchers within/outside the European Economic Area. All data transferred out of the UK/EU is protected under GDPR.

20. Informing your General Practitioner (GP)

Your General Practitioner will be notified of your participation in the study, along with any other practitioners who are involved with your medical care. Please let us know if you do not wish for this information to be disclosed and we will not inform them.

20. What will happen to any samples I give?

Samples of blood, sputum and urine which are taken will be anonymized and will be stored. These will have further analysis to look for markers of infection and may involve culture of the bugs and looking at the genes of any bugs in the sputum with the aim of developing new treatment. This may include analysis and processing at different specialist sites including North America and Europe that can carry out the specific tests. No identifiable data will be transferred to other sites as it will all be anonymized. If there is any biological material left, this will be stored in our laboratory for future testing if necessary.

21. Will any Genetic testing be done?

There are no current plans to do genetic testing. But, samples may be used for future studies into factors that influence bronchiectasis, including genetic studies.

22. What will happen to the results of this clinical trial?

The results of the study will be available after it finishes and will usually be published in a medical journal or be presented at a scientific conference. The data will be anonymous and none of the patients involved in the trial will be identified in any report or publication.

Should you wish to see the publication, please ask your study doctor.

23. Who is organising and funding this clinical trial?

The Nottingham University Hospitals NHS Trust will act a sponsor the research. The Nottingham Biomedical Research Centre will fund the research.

Appendix 4: Consent form

IRAS ID: 235786

Participant Consent Form

Version: 1.2 Date: 23.05.2018

Airway infection in bronchiectasis patients

Principal Investigator: Dr Helen Barr

Patient Study ID: …… Initials: ……

Patient initial each box

1. I confirm that I have read and understand the information sheet dated **23.05.2018 (version1.2)** for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐
3. I understand that the sputum production and blowing test in this study are optional and I can decline these tests if I wish. ☐
4. I understand that my medical records may be looked at by authorised individuals from the Sponsor for the study and the UK Regulatory Authority in order to check that the study is being carried out correctly. ☐
5. I understand that even if I withdraw from the above study, the data and samples collected from me will be used in analysing the results of the trial, unless I specifically withdraw consent for this. ☐
6. I consent to the storage, including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. ☐
7. I agree that my GP, or any other doctor treating me, will be notified of my participation in this study. ☐
8. I agree to take part in the study. ☐

Name of the patient *(Print)*	date	Patient's signature
Name of person taking consent *(Print)*	date	Signature

Original to be retained and filed in the site file. 1 copy to patient, 1 copy to be filed in patient's notes.

Appendix 5: GP letter

Nottingham University Hospitals NHS
NHS Trust

Nottingham Respiratory Research Unit
Clinical Sciences Building
City Hospital
Hucknall Road
Nottingham
NG5 1PB

Tel: 0115 82 31709

Head of division:
Professor AJ Knox

Address:
Date:

Dear Doctor,

Re:

The above patient of yours is participating in a study looking at infection markers associated with acute pulmonary exacerbations of bronchiectasis. This includes taking baseline data and sending sputum, urine and blood samples both in a pulmonary exacerbation and at clinical stability.

If you require any more information, please contact me using the contact details in this letter.

Yours sincerely,

Dr K Webb
Clinical Research Fellow
Microbiology/Infectious diseases Registrar
Email: Karmel.webb@nottingham.ac.uk
Tel: 0115 8231753

Appendix 6: Case report form

NOTTINGHAM BIOMEDICAL
RESEARCH CENTRE

NHS
National Institute for
Health Research

AIRWAY INFECTION IN BRONCHIECTASIS PATIENTS: A FEASIBILITY STUDY

CASE REPORT FORM: Version 1.0 May 2018

Study Purpose

To explore the feasibility of collecting and analysing samples to investigate the microbiological causes of pulmonary exacerbations in patients with bronchiectasis.

Short Title:
Infection and Bronchiectasis

Acronym:
AIB

Study Type:
Cohort

NUH Ref:
17RM028

Chief Investigator:

Dr Helen Barr: helen.barr@nuh.nhs.uk

Study Lead Researcher & Co-Investigator:

Dr Karmel Webb: karmel.webb@nuh.nhs.uk

Co-Investigators:

Dr Andrew Fogarty: andrew.fogarty@tingham.ac.uk

Prof Alan Knox: alan.knox@nottingham.ac.uk

Prof Miguel Camara: Miguel.camara@nottingham.ac.uk

Sponsor:

Nottingham University Hospitals NHS Trust

Funded by:

Nottingham

Biomedical Research Centre

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PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

PATIENT DETAILS

TO BE REMOVED AT THE END OF THE

NHS No:	
NUH K Number	
Date of Birth (dd/mm/yyyy)	

TITLE (circle)	Mr / Mrs / Miss / Ms / Dr / Prof
First Name	
Surname	

Contact Details

Address	
Town	
City	
Post Code	
Telephone	
Mobile	
Email	

GP Details

GP Name	
GP Practice	
Address	
Town	
City	
Post Code	
Tel	

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

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NOTTINGHAM BIOMEDICAL RESEARCH CENTRE

NHS National Institute for Health Research

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

1st REVIEW PATIENT PROFORMA

INCLUSIION CRITERIA	YES	NO
Diagnosis of Bronchiectasis		
Age 16 or over		
Able to give informed consent		

EXCLUSIION CRITERIA	YES	NO
Age Less than 16		
Unable to give informed Consent		
Clinician feels taking part in research is inap-propriate		
Receiving terminal care		

Data collected at Clinical Stability	
Data collected at Acute Exacerbation	

	DD / MM / YYYY
Date of Consent	/ /
Date of Birth	/ /

	M	F
Gender		
AGE (Years)		

ETHNICITY	
White	
Black	
Asian	
Mixed	

SMOKING HISTORY	
Current	
Ex	
Never	
Passive Exposure	

If Ex Smoker; How Long since Stopped (Years)	
Pack Years (Number)	

Height (cm)	
Weight (Kg)	

DIAGNOSIS	DD / MM / YYYY
Date diagnosed with Bronchiectasis (if known)	/ /
Year if actual date not known	

CAUSE OF BRONCHIECTASIS

Unknown	
Immune Deficiency	
ABPA	
NTM	

Rheumatoid Arthritis	
Inflammatory Bowel Disease	
GORD	

Post infective (history of significant childhood infection such as:

Whooping cough	
Tuberculosis	
Measles	
Pneumonia	

Others (list below)

Investigator Name		Investigator Signature		Date (DD/ MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

Historical Microbiology

List sputum microbiology over the last 12 months (most recent 1st)

1	Bacteria									
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non -mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

2	Bacteria									
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non -mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

3	Bacteria									
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non -mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

4	Bacteria									
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non -mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

Historical Microbiology

List sputum microbiology over the last 12 months (most recent 1st)

5	Bacteria									
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non -mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

6	Bacteria									
Date (dd/mm/	Haem	PA:	PA: non	PA:	Strept	Moraxella	Straph	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

7	Bacteria									
Date (dd/mm/	Haem	PA:	PA: non	PA:	Strept	Moraxella	Straph	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

8	Bacteria									
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non -mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

Historical Microbiology

List sputum microbiology over the last 12 months (most recent 1st)

9	Bacteria									
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non -mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

10	Bacteria									
Date (dd/mm/	Haem	PA:	PA: non	PA:	Strept	Moraxella	Straph	MRSA	GNE	Other (state)
Sensivities/ Resistance List										

	Y	N
Is the patient chronically colonised?		

	Y	N
Allergy/ADR?		

List Allergies	

Defined by isolation of a potentially pathogenic bacteria in sputum culture on two or more occasions, at least 3 months apart in a 1 year period,

Chronic Colonisation with?	Bacteria	Year

IV Antibiotic therapy in the last 12 months (only include oral if in last 4 weeks)

Start Date(dd/mm/yyyy)	End Date (dd/mm/yyyy)	Antiobiotic

Start Date(dd/mm/yyyy)	End Date (dd/mm/yyyy)	Antiobiotic

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

BRONCHIECTASIS SEVERITY INDEX

TO BE CALCUATED AT CLINICAL STABILITY

Age (Years)		Hospital Admissions in the past 2 years	
Weight (Kg)		Exacerbation frequency in the last 12 months	
Height (cm)		Colonisation: NOT COLONISED	
BMI (Kg/m2)		Colonisation: Colonised with P.A	
FEV_1 % Predicted		Radiological Severity: < 3 lobes involved	
		Radiological Severity: >=3 lobes or cystic changes	
		MRC Dyspnoea score (1-5)	
		Calculated BSI Score	

Bronchiectasis Aetiology and Co-morbidity Index

Metastatic malignancy		Iron deficiency anaemia	
Haematological malignancy		Diabetes	
COPD		Asthma	
Cognitive impairment		Pulmonary hypertension	
Inflammatory bowel disease		Peripheral vascular disease	
Chronic liver disease		Ischemic Disease	
Others Co-morbidities (list below)		Calculated BACI Score	

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

Concomitant Medications

Medication (Trade or Generic name)	Dose	Units (mg, mcg, ul etc.)	Frequency	Date Started (dd/mm/yyyy)	OR >3 Months if not known (tick)	Date Stopped (dd/mm/yyyy)

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

Further notes/comments

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

INITIAL EXACERBATION PROFORMA

Demographic Baseline Data Completed	

There is a deterioration in three or more of the following key symptoms for at least 48 hours:

Cough		Breathlessness and / or exercise tolerance	
Sputum volume and /or consistency		Fatigue and / or malaise	
Sputum purulence		haemoptysis	

A clinician has determined that a change in bronchiectasis and IV antibiotic treatment is required	

ANTIBIOTICS PRESCRIBED

Antibiotic	Dose	Frequency	Start Date(dd/mm/yyyy)	Start Time	Proposed Duration (days)	End Date (dd/mm/yyyy)

Recent sputum culture results (if not on baseline demographic form)

1	Bacteria										
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non-mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	RCO	Other (state)
Sensivities/ Resistance List											

2	Bacteria										
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non-mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	RCO	Other (state)
Sensivities/ Resistance List											

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

3	Bacteria										
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non-mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	RCO	Other (state)
Sensivities/ Resistance List											

ABX prescribed for exacerbation including recent oral Antibiotics in the last 4 weeks

Antibiotic	Dose	Frequency	Start Date(dd/mm/yyyy)	Start Time	Proposed Duration (days)	End Date (dd/mm/yyyy)

CHECKLIST

Patient has completed Resp Qol-B?		Resp Qol-B Score	
Spontaneous Sputum		Date/Time	
Induced Sputum		Date/Time	
EDTA Blood		Date/Time	
Urine		Date/Time	
Inflammatory Markers		Date/Time	

Port	Peripheral	PICC

Spirometry performed at acute exacerbation

	Predicted Value	Actual Value
FEV1 (L)		
FVC (L)		
PEFR (L/min)		
FEV1/FVC Ratio		

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

Blood Results							
Hb (g/L)	WBC $x10^9$/L	Platelets $x10^9$/L	Neuts $x10^9$/L	Lymphs $x10^9$/L	Eos $x10^9$/L	CRP mg/dL	Creatinine (µmol/L)

Investigator Name		Investigator Signature		Date (DD/ MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

POST EXACERBATION PROFORMA

	Yes	No
Patient has successfully completed IV antibiotic treatment		

ANTIBIOTICS GIVEN

Antibiotic	Dose	Frequency	Duration (Days)	Different to that prescribed (Y/N)	Reason Changed

Any new microbiological results from last review? (If Known)

1	Bacteria										
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non-mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	RCO	Other (state)
Sensivities/ Resistance List											

2	Bacteria										
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non-mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	RCO	Other (state)
Sensivities/ Resistance List											

Investigator Name		Investigator Signature		Date (DD/ MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

BLOOD CULTURES

Date (dd/mm/yyyy)	Organisms

VIRAL THROAT SWAB

Date (dd/mm/yyyy)	Organisms

Patient has completed Resp Qol-B?		Resp Qol-B Score	
Spontaneous Sputum		Date/Time	
Induced Sputum		Date/Time	
EDTA Blood		Date/Time	
Urine		Date/Time	
Inflammatory Markers		Date/Time	

Port	Peripheral	PICC

Spirometry performed at acute exacerbation

	Predicted Value	Actual Value
FEV1 (L)		
FVC (L)		
PEFR (L/min)		
FEV1/FVC Ratio		

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

Blood Results							
Hb (g/L)	WBC $x10^9$/L	Platelets $x10^9$/L	Neuts $x10^9$/L	Lymphs $x10^9$/L	Eos $x10^9$/L	CRP mg/dL	Creatinine (µmol/L)

Investigator Name		Investigator Signature		Date (DD/ MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

STABILITY REVIEW PROFORMA

BASELINE DATA COMPLETED	Yes	No
Initial Exacerbation		
1st Review at Clinical Stability		

THIS REVIEW IS	Yes	No
1st Patient Review at Clinical Stability		
Post Exacerbation Clinical Stability		

Patient does NOT fulfil the criteria for acute exacerbation		
Patient has not received IV antibiotics in the last 4 weeks		

Any new microbiological results NOT included on BASELINE DATA or EXCERBATION PROFORMA

1	Bacteria										
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non-mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	RCO	Other (state)
Sensivities/ Resistance List											

2	Bacteria										
Date (dd/mm/yyyy)	Haem Inf	PA: Mucoid	PA: non-mucoid	PA: unknown	Strept Pneu	Moraxella cat	Straph aureas	MRSA	GNE	RCO	Other (state)
Sensivities/ Resistance List											

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

BLOOD CULTURES

Date (dd/mm/yyyy)	Organisms

VIRAL THROAT SWAB

Date (dd/mm/yyyy)	Organisms

CHECKLIST

Patient has completed Resp Qol-B?		Resp Qol-B Score	
Spontaneous Sputum		Date/Time	
Induced Sputum		Date/Time	
EDTA Blood		Date/Time	
Urine		Date/Time	
Inflammatory Markers		Date/Time	

Port	Peripheral	PICC

Spirometry performed at acute exacerbation

	Predicted Value	Actual Value
FEV1 (L)		
FVC (L)		
PEFR (L/min)		
FEV1/FVC Ratio		

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

Blood Results							
Hb (g/L)	WBC $x10^9$/L	Platelets $x10^9$/L	Neuts $x10^9$/L	Lymphs $x10^9$/L	Eos $x10^9$/L	CRP mg/dL	Creatinine (µmol/L)

EXACERBATIONS

Number of Exacerbations (Up to 01/11/2020)	

Further notes/comments

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

PARTICIPANT UNIQUE STUDY ID	INITIALS	DOB (DD/MM/YYYY)
AIB		/ /

END OF STUDY	YES	NO		
Patient Completed the Study			Date Completed (dd/mm/yyyy)	
Patient Withdrew and Did not Complete the Study			Date of Withdrawal (dd/mm/yyyy)	

REASON FOR WITHDRAWAL	YES	NO		
Withdrawal of Consent				
Loss to Follow Up				
Death			Date of Death (dd/mm/yyyy)	
Protocol Violation				
Other			Cause of Death (if known)	

Investigator Name		Investigator Signature		Date (DD/MM/YYYY)	/ /

Appendix 7: Quality of life questionnaire

QOL-B QUALITY OF LIFE QUESTIONNAIRE – BRONCHIECTASIS

Understanding the impact of your illness and treatments on your everyday life can help your doctor monitor your health and adjust your treatments. For this reason, we have developed a quality of life questionnaire specifically for people who have bronchiectasis. Thank you for your willingness to fill in this questionnaire.

Instructions: The following questions are about the current state of your health, as you perceive it. This information will allow us to better understand how you feel in your everyday life.

Please answer all the questions. There are **no** right or wrong answers! If you are not sure how to answer, choose the response that seems closest to your situation.

Demographics

Please fill in the information or tick the box to indicate your answer.

A. What is your date of birth?

Date ☐☐/☐☐/☐☐☐☐
Day Month Year

B. What is your gender?

☐ Male ☐ Female

C. During the **past week**, have you been on holiday or not studying or working for reasons **NOT** related to your health?

☐ Yes ☐ No

D. What is your current marital status?

☐ Single/never married
☐ Married
☐ Widowed
☐ Divorced
☐ Separated
☐ Remarried
☐ Living with a partner

E. Which of the following best describes your ethnic group?

☐ White
☐ Mixed/multiple ethnic groups
☐ Asian/Asian British
☐ Black/African/Caribbean/Black British
☐ Other (please describe) _____
☐ Prefer not to answer this question

F. What is the highest level of education you have completed?

☐ Some secondary school or less
☐ GCSEs/Standard Grades or equivalent
☐ A Level/Higher/Advanced Higher or equivalent
☐ Some college or university
☐ College qualification (e.g. HNC, HND, Foundation Degree)
☐ Undergraduate degree (e.g. BA, BSc)
☐ Postgraduate degree (e.g. MA, MSc, PhD)

G. Which of the following best describes your current work or educational status?

☐ Studying outside the home
☐ Studying at home/distance learning
☐ Seeking work
☐ Working full-time or part-time (either outside the home or at a home-based business)
☐ Full-time housewife/househusband
☐ Not studying or working due to my health
☐ Not working for other reasons/Retired

Continue to Next Page

QOL-B QUALITY OF LIFE QUESTIONNAIRE – BRONCHIECTASIS

Section I. Quality of Life

Please tick a box to indicate your answer.

During the past week, to what extent have you had difficulty:	A lot of difficulty	Moderate difficulty	A little difficulty	No difficulty
1. Performing vigorous activities, such as gardening or exercising	☐	☐	☐	☐
2. Walking as fast as other people (family, friends, etc.)	☐	☐	☐	☐
3. Carrying heavy things, such as books or shopping bags	☐	☐	☐	☐
4. Climbing one flight of stairs	☐	☐	☐	☐

During the past week, indicate how often:	Always	Often	Sometimes	Never
5. You felt well	☐	☐	☐	☐
6. You felt tired	☐	☐	☐	☐
7. You felt anxious	☐	☐	☐	☐
8. You felt energetic	☐	☐	☐	☐
9. You felt exhausted	☐	☐	☐	☐
10. You felt sad	☐	☐	☐	☐
11. You felt depressed	☐	☐	☐	☐

Are you currently on any treatments (such as: oral or inhaled medications; a PEP, Acapella® or Flutter® device; chest physiotherapy; or Vest) for bronchiectasis?

☐ Yes ☐ No (Go to Question 15 on the next page)

Please circle a number to indicate your answer. Please choose only one answer for each question.

12. To what extent do your treatments for bronchiectasis make your daily life more difficult?
    1. Not at all
    2. A little
    3. Moderately
    4. A lot

13. How much time do you currently spend each day on your treatments for bronchiectasis?
    1. A lot
    2. A moderate amount
    3. A little
    4. Almost none

14. How difficult is it for you to fit in your treatments for bronchiectasis each day?
    1. Not at all
    2. A little
    3. Moderately
    4. Very

Continue to Next Page

Please circle a number to indicate your answer. Please choose only one answer for each question.

15. How do you think your health is now?
1. Excellent
2. Good
3. Fair
4. Poor

Please tick a box to indicate your answer.
Thinking about your health during the past week, indicate the extent to which each sentence is true for you.

	Completely true	Mostly true	A little true	Not at all true	Doesn't apply
16. I have to limit vigorous activities, such as walking or exercising	☐	☐	☐	☐	
17. I have to stay at home more than I want to	☐	☐	☐	☐	
18. I am worried about being exposed to other people who are ill	☐	☐	☐	☐	☐
19. It is difficult to be intimate with a partner (kissing, hugging, sexual activity)	☐	☐	☐	☐	☐
20. I lead a normal life	☐	☐	☐	☐	
21. I am concerned that my health will get worse	☐	☐	☐	☐	
22. I think my coughing bothers other people	☐	☐	☐	☐	
23. I often feel lonely	☐	☐	☐	☐	
24. I feel healthy	☐	☐	☐	☐	
25. It is difficult to make plans for the future (holidays, attending family events, etc.)	☐	☐	☐	☐	
26. I feel embarrassed when I am coughing	☐	☐	☐	☐	

Please circle a number or tick a box to indicate your answer.

During the past week:

27. To what extent did you have trouble keeping up with your job, housework, or other daily activities?
1. You have had no trouble keeping up
2. You have managed to keep up but it has been difficult
3. You have been behind
4. You have not been able to do these activities at all

	Always	Often	Sometimes	Never
28. How often does having bronchiectasis get in the way of meeting your work, household, family, or personal goals?	☐	☐	☐	☐

Continue to Next Page

QOL-B QUALITY OF LIFE QUESTIONNAIRE – BRONCHIECTASIS

Section II. Respiratory Symptoms

Please tick a box to indicate your answer.

Indicate how you have been feeling during the past week:	A lot	A moderate amount	A little	Not at all
29. Have you felt congestion (fullness) in your chest?	☐	☐	☐	☐
30. Have you been coughing during the day?	☐	☐	☐	☐
31. Have you had to cough up sputum?	☐	☐	☐	☐

32. Has your sputum been mostly:

☐ Clear ☐ Clear to yellow ☐ Yellowish-green
☐ Brownish-dark ☐ Green with traces of blood ☐ Don't know

How often during the past week:	Always	Often	Sometimes	Never
33. Have you had shortness of breath when being more active, such as when doing housework or gardening?	☐	☐	☐	☐
34. Have you had wheezing?	☐	☐	☐	☐
35. Have you had chest pain?	☐	☐	☐	☐
36. Have you had shortness of breath when talking?	☐	☐	☐	☐
37. Have you woken up during the night because you were coughing?	☐	☐	☐	☐

Please make sure you have answered all the questions.

THANK YOU FOR YOUR COOPERATION!

Appendix 8: Research

JOURNAL OF MEDICAL MICROBIOLOGY

SHORT COMMUNICATION

Webb et al., Journal of Medical Microbiology 2019;68:1823–1828

DOI 10.1099/jmm.0.001099

MICROBIOLOGY SOCIETY

Clinical significance of *Pseudomonas aeruginosa* 2-alkyl-4-quinolone quorum-sensing signal molecules for long-term outcomes in adults with cystic fibrosis

Karmel Webb[1,*], Andrew Fogarty[1], David A. Barrett[2], Edward F. Nash[3], Joanna L. Whitehouse[3], Alan R. Smyth[4], Iain Stewart[5], Alan Knox[5], Paul Williams[6], Nigel Halliday[6], Miguel Cámara[6] and Helen L. Barr[7]

Abstract

Introduction. *Pseudomonas aeruginosa* is an important respiratory pathogen in cystic fibrosis (CF), which is associated with an accelerated decline in lung function, frequent pulmonary exacerbations and increased mortality. *P. aeruginosa* produces intercellular signalling molecules including 2-alkyl-4-quinolones (AQs), which regulate virulence-factor production and biofilm formation in the CF airways. Studies have shown that AQs are detectable in the sputum and plasma of adults with CF and chronic pulmonary *P. aeruginosa*.

Aim. We tested the hypothesis that the presence of six AQs in plasma or sputum obtained from adults with CF was associated with long-term adverse clinical outcomes.

Methodology. We analysed clinical data over an 8year follow period for 90 people with CF who had previously provided samples for AQ analysis at clinical stability. The primary outcome was all cause mortality or lung transplantation. Secondary outcomes were the rate of lung-function decline and the number of intravenous (IV) antibiotic days for pulmonary exacerbations.

Results. There was no statistical association between the presence of any of the six measured AQs and the primary outcomes or the secondary outcome of decline in lung function. One of the six AQs was associated with IV antibiotic usage. The presence of 2-nonyl-3-hydroxy-4(1h)-quinolone (C9-PQS) in sputum was associated with an increase in the number of IV antibiotic days in the follow-up period (Mann–Whitney; P=0.011).

Conclusion. Further investigation to confirm the hypothesis that C9-PQS may be associated with increased antibiotic usage for pulmonary exacerbations is warranted as AQ-dependent signalling is a potential future target for anti-virulence therapies.

INTRODUCTION

Pseudomonas aeruginosa is a highly successful opportunistic Gram-negative bacterium, which is well adapted to the airway niche in cystic fibrosis (CF). *P. aeruginosa* is the dominant pathogen in the CF lung and is associated with increased morbidity and mortality in this population [1]. *P. aeruginosa* is intrinsically resistant to many classes of antibiotics, produces a host of virulence factors and forms impenetrable biofilms in the CF airways [2]. *P. aeruginosa* controls the production of these virulence factors using a cell-to-cell communication known as quorum sensing (QS) [3]. This allows the whole bacterial population to sense and respond to changes in environmental stimuli and to coordinate gene expression of the community as a whole.

The *P. aeruginosa* QS system consists of three interlinking QS circuits, one of which is the *pqs* QS circuit. The *pqs* QS

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Abbreviations: *P. aeruginosa*, *Pseudomonas aeruginosa*.
Two supplementary tables and one supplementary figure are available with the online version of this article.

systems uses multiple 2-alkyl-4 quinolones (AQs) as signal molecules, including the pseudomonas quinolone signal (PQS) molecule [2-heptyl-3-hydroxy-4(1 h)-quinolone] and its precursor HHQ (2-heptyl-4-hydroxyquinoline) [4]. Both PQS and HHQ act as autoinducers to increase AQ biosynthesis [5]. In addition, PQS is crucial for the production of virulence factors and biofilm formation both *in vitro* and in animal models of infection [5]. *P. aeruginosa* produces over 50 AQs, and the roles of many of these AQs are not yet fully understood [6].

Several AQs are detectable in the sputum and plasma of adults with CF and chronic pulmonary *P. aeruginosa*. Higher systemic concentrations of several AQs are associated with higher *P. aeruginosa* loads and lower lung function, in cross-section analyses [7, 8]. This suggests that high systemic AQ levels may be associated with an adverse prognosis. In addition, systemic concentrations of several AQs decrease following intravenous anti-pseudomonal antibiotics [8], suggesting they have potential as biomarkers of change in clinical status.

We hypothesized that higher levels of AQs in the sputum and plasma of adults with CF would be associated with adverse long-term clinical outcomes in this patient population.

We investigated whether detection of six individual AQs in the sputum and plasma of people with CF could be linked to long-term outcomes, including death or lung transplantation, as well as the annual rate of lung function decline and intravenous (IV) antibiotic use for pulmonary exacerbations. Clinical data over an 8 year period was retrospectively collected on 90 adults with CF who had previously participated in an AQ biomarker study [7].

METHODS

Participants

We studied 90 adults with CF who had previously participated in an AQ biomarker study, the full details of which were previously published [7]. In summary, participants were recruited at clinical stability from two UK adult CF centres between the years 2009 and 2011. Baseline demographic data and data on six AQs measured in both sputum and plasma samples were used [7].

Study design

Follow-up clinical data were retrospectively obtained from the UK CF registry. Annual data were collected from the participants from the year of recruitment to the end of the study period in 2017. Data on death, lung transplantation, lung function and the number of IV antibiotic days for pulmonary exacerbations were obtained. The number of IV antibiotic days was measured annually from the year of recruitment to the end of 2017. For lung function data, the best recorded forced expiratory volume in 1 s (FEV_1) of the preceding year was used.

The primary outcomes were death or lung transplantation during the follow-up period. Secondary outcomes were the number of IV antibiotic days for pulmonary exacerbations and the rate of decline in FEV_1.

Sample processing and AQ analysis

All sample processing and AQ analyses were performed in the initial study as previously described [7]. Sputum plugs were harvested for quantitative AQ analyses [9, 10]. Venous blood samples were centrifuged at 1000 ***g*** for 15 min at 4 °C, plasma was then separated and snap frozen in liquid nitrogen. Sputum samples for AQ analysis were extracted using acidified ethyl acetate (Fisher Chemicals, Loughborough, UK) [9, 10]. Plasma samples were extracted by solid-phase extraction and plasma matrix-matched samples from a healthy volunteer donor were prepared to allow calibration of samples. Prepared clinical samples for AQ analyses were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Ortori *et al.* [9]. All samples were analysed once using LC-MS/MS with no replicate analysis performed.

The lower limit of quantification (LLOQ) was established by using serial dilutions of the analyte mix and spiking into blank plasma samples prior to extraction and analysis, The LLOQ for plasma was defined as the analyte concentration at which a signal/noise ratio of 10:1 was achieved. In the absence of blank sputa to produce matrix-matched calibration, 1.0 ml aliquots of 0.9% NaCl were used and there was no LLOQ defined for sputum samples. A total of six AQs were analysed individually: HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline), PQS [2-heptyl-3-hydroxy-4(1 h)-quinolone], C9-PQS [2-nonyl-3-hydroxy-4(1 h)-quinolone], HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) and NQNO (2-nonyl-4-hydroxyquinoline-*N*-oxide). Calculated LLOQs in plasma samples were as follows: HHQ 10 pmol l^{-1}; NHQ 10 pmol l^{-1}; HQNO 30 pmol l^{-1}, NQNO 40 pmol l^{-1}; PQS 100 pmol l^{-1} and C9-PQS 100 pmol l^{-1} [7]. Quantitative concentrations for the six AQs both in plasma and sputum are summarized in Table S1 (available in the online version of this article).

Statistical analysis

The six measured AQs were analysed individually and combined detectable AQ levels in sputum and plasma were calculated.

For initial analyses, individual AQ levels were classified as detectable or undetectable (concentrations above or below the LLOQ, respectively). A binary measure of combined AQs was defined as a detection of at least one individual AQ in sputum or plasma, respectively. Two-sample Wilcoxon rank-sum (Mann–Whitney) tests and Pearson's chi-squared tests were used to assess binary AQ levels with the primary and secondary clinical outcomes.

If significant associations were obtained using binary AQ analyses, further quantitative analyses were performed. Individual AQ concentrations were then compared with

Table 1. Baseline clinical characteristics and *P. aeruginosa* status of participants

Variable	Baseline (*n*=83)
Nottingham University Hospitals NHS trust	42
University Hospitals Birmingham NHS Foundation Trust	41
Age in years: median (range)	28.4 (17.8 to 61.5)
Gender, males (%)	54 (65.1)
FEV_1 % predicted: mean (SD)	58 (±20)
Absolute FEV_1 in L: mean (SD)	2.13 (±0.9)
BMI: mean (SD)	22.9 (±3.3)
P. aeruginosa status at baseline: *n* (%)	
Never	0 (0)
Free	1 (1.2)
Intermittent	2 (2.4)
Chronic	80 (96.4)

P. aeruginosa status of participants defined by Leeds criteria [29].
n, number of participants with data available; SD, standard deviation.

Table 2. Summary of clinical data during the follow-up period.

Variable	*N*	(%)	Outcome
Follow-up time†	83	100.0 %	6.3 (5.6–6.7)
No. of deaths/lung transplantation	23	27.7 %	
Died during follow up	15	18.1 %	
Rate of decline per year‡:			
Absolute FEV_1 (ml)	80	96.4 %	53.1 (55.5)
Percent predicted FEV_1 (%)	80	96.4 %	1.6 (2.0)
No. of IV antibiotic days per year*:			
Overall	81	97.6 %	37.5 (16.4–58.7)
No death/transplant	59	71.1 %	31.9 (13.0–43.7)
Death/transplant*	22	26.5 %	60.1 (46.5–80.4)

†, reported as median and interquartile range. ‡, reported as mean and SD. *Mann–Whitney significance $P<0.001$. IV, intravenous. *N*, number of participants with data available.

clinical outcomes using Spearman rank correlation coefficients. Statistical significance was assessed as $P<0.05$. All data were analysed using Stata SE15 statistical software (TX, USA).

RESULTS

Of the 90 participants in the original study, seven were lost during follow up and therefore 83 participants were included in the analyses. Baseline characteristics are summarized in Table 1.

The median follow-up period was 6.3 years (IQR 5.6 to 6.7 years). During the follow up period, 23 participants (27 %) either died or had bilateral lung transplantation. Three people had a bilateral lung transplant and subsequently died during the follow-up period. These characteristics are summarized in Table 2.

Presence or absence of detectable levels of AQs at baseline on primary outcomes

Death or lung transplantation during follow up was not statistically different in the presence or absence of detectable levels of six individual AQs at baseline (Table 3), using binary AQ analyses (detected versus not detected). Similarly, there were no statistical associations between the combined AQs measured and primary outcomes, indicating indicating independence (chi-squared $P=0.751$ and $P=0.351$ for total sputum and plasma AQs, respectively, Table 3).

The presence or absence of detectable levels of AQs at baseline on secondary outcomes

There was no statistical difference demonstrated with the rate of FEV_1 decline both with individual and combined AQs detected in plasma or sputum (Table S2).

There was no association between five of the individual AQs and the number of IV antibiotic days per year (Table S2).

The presence of C9-PQS in the sputum was associated with an increase in IV antibiotic days per year during the follow-up period (Mann–Whitney $P=0.011$; Fig. 1). The median number of IV antibiotic days per year if sputum C9-PQS was detected was 41.4 (IQR: 26.6 to 60.7) compared with 28.2 (IQR: 4.0 to 44.4) when not detected. A similar finding was observed when follow up was restricted to 3 years; 44.8 (IQR: 25.1 to 62.1) when sputum C9-PQS was detected compared with 29.7 (IQR: 4.7 to 50.4) when not detected (Mann–Whitney $P=0.046$). The concentration of C9-PQS in sputum was positively correlated with the number of IV antibiotics per year but did not reach statistical significance (Spearman rank correlation: $r=0.2$, $P=0.09$). There was no statistical difference in the number of IV antibiotic days when C9-PQS was detected in plasma (Table S1; Mann–Whitney $P=0.32$), nor detectable levels of total combined AQs in plasma or sputum (Table S1). The number of IV antibiotic days per year was statistically higher in people who died or had a bilateral lung transplantation in the follow-up period (Mann–Whitney $P<0.001$, Table 2).

Table 3. Individual and combined total AQs with primary outcome of death or lung transplantation

AQ	Death/transplant: *n* (%)	No death/ transplant: *n* (%)	*P*-value (chi²)
Sp HHQ +	14 (70)	34 (65.4)	0.71
Sp HHQ –	6 (30)	18 (34.6)	
Pl HHQ +	14 (66.7)	35 (58.3)	0.501
Pl HHQ –	7 (33.3)	25 (41.7)	
Sp NHQ +	15 (75)	35 (67.3)	0.526
Sp NHQ –	5 (25)	5 (32.7)	
Pl NHQ +	3 (14.3)	18 (30)	0.157
Pl NHQ –	18 (85.7)	42 (70)	
Sp PQS +	12 (60)	31 (59.6)	0.98
Sp PQS –	8 (40)	21 (40.4)	
Pl PQS +	8 (38.1)	20 (33.3)	0.693
Pl PQS –	13 (61.9)	40 (66.7)	
Sp C9-PQS +	15 (75)	33 (63.5)	0.352
Sp C9-PQS –	5 (25)	19 (36.5)	
Pl C9-PQS +	3 (14.3)	5 (8.3)	0.431
Pl C9-PQS –	18 (85.7)	55 (91.7)	
Sp HQNO +	16 (80)	39 (75)	0.655
Sp HQNO –	4 (20)	13 (25)	
Pl HQNO +	11 (52.4)	28 (46.7)	0.652
Pl HQNO –	10 (47.6)	32 (53.3)	
Sp NQNO +	14 (70)	39 (75)	0.666
Sp NQNO –	6 (30)	13 (25)	
Pl NQNO +	8 (38.1)	21 (35)	0.799
Pl NQNO –	13 (61.9)	39 (65)	
Total Sp AQ +	18 (90)	48 (92.3)	0.751
Total Sp AQ –	2 (10)	4 (7.7)	
Total Pl AQ +	15 (71.4)	36 (60)	0.351
Total Pl AQ –	6 (28.6)	24 (40)	

+, detected; –, undetected; Sp, Sputum; Pl, Plasma; AQ, 2-alkyl-4 quinolones; *n*, number; HHQ, 2-heptyl-4-hydroxyquinoline; NHQ, 2-nonyl-4-hydroxyquinoline; PQS, 2-heptyl-3-hydroxy-4(1 h)–quinolone; C9-PQS, 2-nonyl-3-hydroxy-4(1 h)-quinolone; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO, 2-nonyl-4–hydroxyquinoline-*N*-oxide.

DISCUSSION

This is the first study to explore the relationship between baseline AQ quorum-sensing signal molecules measured in sputum or plasma with long-term outcomes in adults with CF. There were no significant differences between the detection of sputum or plasma AQs at baseline with death, lung transplantation or rate of FEV_1 decline over the follow-up period. One of the six AQs measured was associated with increased IV antibiotic usage in the follow-up period. A higher number of IV antibiotic days for pulmonary exacerbations were observed in the presence of detectable levels of sputum C9-PQS. However, plasma levels of C9-PQS levels were not significantly associated with IV antibiotic usage. This may be explained by the low number of participants with detectable C9-PQS in plasma (eight adults compared to 48 adults with detectable C9-PQS in sputum).

There are a number of limitations in this study that should be considered when interpreting these data. This is a retrospective analysis and the number of participants who died or had lung transplantation resulted in small numbers. We primarily assessed AQ levels as dichotomous; detectable or not detectable, as the variability across the sample size would have provided low power. Intra-subject variability of AQ concentrations is unknown and a single measure of AQ concentration may not reflect the longer period during which the AQs may influence disease progression. Whilst effect sizes are robust, findings are to be regarded as 'hypothesis generating' as significant *P*-values may be a consequence of multiple hypothesis testing.

Despite limitations, these findings provide evidence of a possible association between both sputum and plasma C9-PQS levels and antibiotic usage, which should be confirmed through prospective study design.

P. aeruginosa is the major respiratory pathogen in people with CF and is difficult to eradicate from the CF airways as it is intrinsically resistant to many classes of antibiotics and forms antibiotic-resistant biofilms [11]. The AQ class of quorum-sensing molecules plays an important role in pathogenicity for *P. aeruginosa* and AQ-deficient mutants show reduced virulence in infection models [12–14]. PQS and its immediate precursor HHQ are the major AQ signalling molecules in *P. aeruginosa* [15]. PQS regulates the expression of at least 182 genes including those that code for the iron-chelating siderophores, pyoverdine and pyochelin [16, 17] as well as playing a role in regulating antibiotic resistance and biofilm maturation [18–20]. In addition, PQS regulates the production of key virulence factors that are associated with pulmonary exacerbations such as elastase [21], pyocyanin [22] and cyanide [23]. Both C9-PQS and NHQ are as effective as their C7 congeners PQS and HHQ at activating the AQ receptor PqsR, which further drives the autoinduction of AQ biosynthesis and up regulates key virulence determinants [24]. Furthermore, molecular-imaging techniques have shown that initial biofilm formation is marked by a dramatic increase in the production of C9-PQS, suggesting it may be important for the growth of *P. aeruginosa* in communities and early biofilm formation [25]. Although our current understanding of the role of C9-PQS is limited [26], it is biologically plausible that C9-PQS may be associated with increased antibiotic usage due to increased virulence-factor production during pulmonary exacerbations.

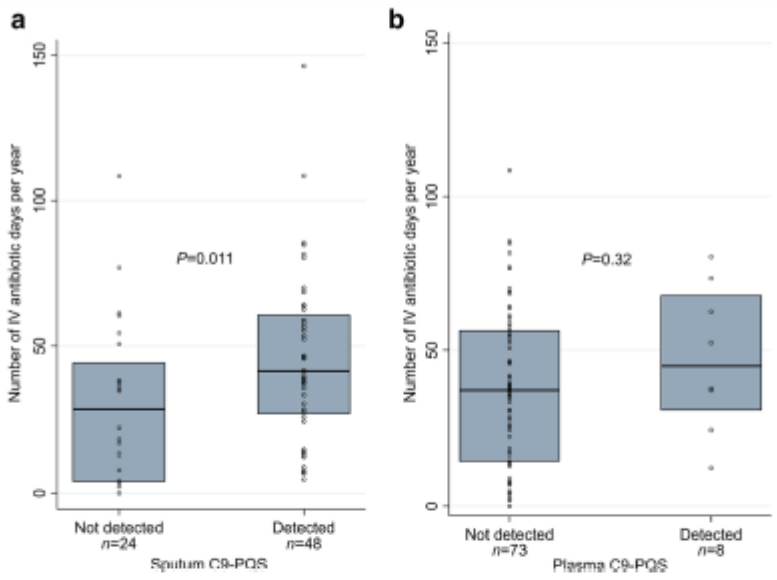

Fig. 1. Annual number of IV antibiotic days according to C9-PQS detection at baseline. Box plot showing the relationship between the presence and absence of detectable levels of sputum and plasma C9-PQS at baseline with the number of intravenous antibiotics days per year during the follow-up period. Box represents interquartile range, line represents median value. C9-PQS=2-nonyl-3-hydroxy-4(1 h)-quinolone; IV=Intravenous; *P*-value derived from Mann–Whitney test; *n*=number of observations.

Recurrent severe pulmonary exacerbations are associated with both increased morbidity and mortality in CF [27]. In recent years, attempts to develop new classes of antimicrobial agents have included targeting of virulence factors or virulence regulatory mechanisms. Consequently, the AQ-signalling system is a promising potential target for antimicrobial agents, which do not kill the organism but instead block or attenuate the ability to cause disease. This is important as multiple courses of antibiotics are detrimental to the host and contribute to a growing global burden of multi-antibiotic resistance that needs to be addressed urgently.

In conclusion, this hypothesis-generating study showed an association between C9-PQS detected in the sputum and increased antibiotic usage in the CF population, which requires more comprehensive investigation to confirm or refute these findings. However, there were no other associations between the five AQs detected and adverse clinical outcomes measured. There is much to learn about AQ regulation in the clinical setting, particularly as development of anti-virulence drugs that target PQS-dependent QS pathways progresses [28].

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Author contributions
The study was designed by K.W./H.B./A.F. K.W. collected the data. The analysis was performed by K.W. and I.S. All authors contributed to data interpretation, data presentation and writing of the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest
The University of Nottingham has a patent for the use of alkyl quinolones as biomarkers for *P. aeruginosa* infection (PCT/GB2014/051458).

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Short communication

Novel detection of specific bacterial quorum sensing molecules in saliva: Potential non-invasive biomarkers for pulmonary *Pseudomonas aeruginosa* in cystic fibrosis

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Quorum sensing

ABSTRACT

Pseudomonas aeruginosa produces specific signalling molecules, 2-alkyl-4-quinolones (AQs) that are detectable in the sputum of adults with cystic fibrosis (CF) and who have pulmonary infection with this opportunistic pathogen. This study aimed to determine whether AQs could be detected in saliva of patients with CF and known infection with *Pseudomonas aeruginosa*. Saliva and sputum samples were obtained from 89 adults with CF and analyzed using liquid chromatography-tandem mass spectrometry. AQs were detected in 39/89 (43.8%) saliva samples and 70/77(90.9%) sputum samples. Salivary AQs had a sensitivity of 50% (95%CI; 37.8; 62.2), specificity of 100% (95%CI; 47.8; 100), when compared to a molecular microbiological measure of *P. aeruginosa* in sputum as measured using polymerase chain reaction. Specific AQs produced by *P. aeruginosa* can be detected in the saliva and warrant investigation as potential non-invasive biomarkers of pulmonary *P. aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is a dominant respiratory pathogen in the cystic fibrosis (CF) lung and is associated with increased morbidity and mortality in this population [1]. Regular microbiological surveillance of the CF airways is important for the early detection of *P. aeruginosa* to facilitate timely and targeted antimicrobial therapy [2]. However, it is difficult to diagnose pulmonary *P. aeruginosa* in people with CF who are unable to spontaneously produce sputum samples. More invasive procedures such as broncho-alveolar lavage or sputum induction are rarely suitable for clinical surveillance programmes. Current clinical practice uses cough swabs, but these lack sensitivity compared to sputum induction and cannot reliably exclude pulmonary *P. aeruginosa* [3]. Finding the best way to diagnose and treat *P. aeruginosa* has also been identified as one of the top 10 research priorities by the James Lind Alliance priority setting partnership in CF [4].

P. aeruginosa uses cell-to-cell signalling systems known as quorum sensing (QS) to regulate the production of biofilms and virulence factors [5,6]. The *pqs* QS system produced by *P. aeruginosa* uses multiple 2-alkyl-4 quinolones (AQs) as signal molecules. Six AQs are detectable in the sputum, plasma and urine of adults with CF and chronic pulmonary *P. aeruginosa* [7,8]. Furthermore, concentrations of three of these AQs; HHQ 2-heptyl-4-hydroxyquinoline (HHQ), 2-nonyl-4-hydroxyquinoline (NHQ) and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), are strongly associ-

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ated with *P. aeruginosa* load measured by routine sputum culture at clinical stability [6].

The aims of this study were to determine whether (i) AQs are detectable in salivary samples obtained from adults with CF with known pulmonary *P. aeruginosa* and (ii) to investigate if salivary AQ concentrations correlate with sputum quantitative load of *P. aeruginosa* measured by polymerase chain reaction (PCR).

2. Methods

2.1. Participants and study design

Eighty-nine adults with CF were recruited at clinical stability to an AQ biomarker study, the full details of which were previously published [7]. In summary, adults aged 16 to 60 were recruited from two UK adult CF centres. All of the participants were known to have previously cultured *P. aeruginosa* from sputum samples and 85 (96%) were chronically infected as defined by the Leed's criteria [9]. Participants known to have previously isolated *Burkholderia cepacia complex* were excluded from the study, as these organisms can also produce AQs [7,10].

2.2. Sample processing

Salivary samples were expelled into universal containers after mouth rinsing with hospital tap water. Spontaneous sputum samples were collected after the salivary samples were obtained according to standard guidelines [11]. Sputum plugs and saliva were stored in duplicate aliquots and frozen at −80 °C, when sufficient volume was available to split the sample. Prepared clinical samples for AQ analyses were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Ortori et al. [12].

A total of six AQs were analysed using LC-MS/MS based on their abundance in CF sputum: HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline), PQS (2-heptyl-3-hydroxy-4(1*H*)-quinolone), C9-PQS (2-nonyl-3-hydroxy-4(1*H*)-quinolone), HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) and NQNO (2-nonyl-4-hydroxyquinoline-*N*-oxide) [5,6].

2.3. Polymerase chain reaction (PCR) analysis

Prior to DNA extraction, sputum samples were pre-treated with propidium monoazide (PMA) to bind dead/compromised bacterial cells [13]. With this modification, only viable (live *P. aeruginosa*) cells are amplified and quantified during the qPCR process [14]. Further details on the AQ and qPCR methodology are provided in the on-line supplement.

2.4. Statistical analysis

AQ concentrations and quantitative load of *P. aeruginosa* were analysed after the addition of 1 to each value followed by $\log_{10}$ transformation to scale the data for interpretation.

The concentrations of AQs in saliva, sputum and *P. aeruginosa* load were compared using Spearman rank correlation coefficients. Sensitivity, specificity, receiver operating characteristic (ROC), positive and negative predicted values of saliva AQs compared to *P. aeruginosa* in sputum measured by PCR were calculated. A McNemar's test was performed to ascertain the agreement of the two sampling techniques. Statistical significance was assessed as $p < 0.05$. All data were analysed using Stata SE15 statistical software (Texas, USA).

3. Results

The majority of participants 85/89 (95.6%) were chronically colonised with *P. aeruginosa* [12]. Baseline demographics are summarised in the online supplement. *P. aeruginosa* was detected in 70/75 (93.3%) sputum samples available for qPCR analysis.

Out of the 6 AQs investigated, at least one AQ was detected in 39/89 (43.8%) saliva samples and 70/77 (90.9%) sputum samples. Salivary AQs had a sensitivity of 50% (95%CI; 37.8;62.2), specificity of 100% (95%CI; 47.8;100), positive predictive value of 100% (95%CI; 90;100), negative predictive of value 12.5% (95%CI;4.2;26.8) compared to live *P. aeruginosa* load in sputum measured using PCR (Table 1).

3.1. Correlation of AQs concentrations measured in saliva and spontaneous sputum

There were significant positive associations between all 6 AQs measured in spontaneous sputum and saliva samples (Table 2 and Fig. 1).

3.2. Correlations between HHQ, NHQ and HQNO levels in saliva with quantitative load of P. aeruginosa in sputum

The quantitative load of *P. aeruginosa* in sputum was significantly correlated with 4 AQs: HHQ ($r = 0.477$, $p < 0.001$), NHQ ($r = 0.374$, $p = 0.001$), HQNO ($r = 0.443$, $p < 0.001$) and NQNO ($r = 0.441$, $p < 0.001$) concentrations in saliva (Table 2 and Fig. 1). The remaining 2 AQs did not reach statistical significance with quantitative *P. aeruginosa* load: C7-PQS ($r = 0.2013$, $p = 0.081$) and C9-PQS ($r = 0.197$, $p = 0.091$).

Fig. 1 legend; Median (interquartile range) for sputum *P. aeruginosa* load by was 7.03 $\log_{10}$CFU/g (6.43 to 7.48) of sputum. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO: 2-nonyl-4-hydroxyquinoline-*N*-oxide, r = Spearman's correlation coefficient.

4. Discussion

This is the first study to examine saliva samples obtained from adults with CF for the presence of AQ quorum signal molecules produced by *P. aeruginosa*. All six AQs were detected in salivary samples and there were positive correlations between concentrations of AQs measured in saliva and corresponding sputum samples. In addition, salivary concentrations of four AQs (HHQ, NHQ, HQNO and NQNO) correlated significantly with quantitative load of sputum *P. aeruginosa* measured using PCR. Strengths of this study include a large multicentre cohort and the pre-treatment of sputum with PMA which enabled only viable *P. aeruginosa* bacterial cells to be amplified and quantified by PCR, providing a precise quantitative measure of the burden of infection.

It is well known that *P. aeruginosa* is highly adapted to the lung environment and highly prevalent in the CF lung. However, diagnosing pulmonary *P. aeruginosa* can be difficult in people who are unable to spontaneously produce sputum. Furthermore, spontaneous sputum production is likely to decrease in the longer term due to the widespread use of highly effective cystic fibrosis transmembrane conductance regulator (CTFR) modulator therapies which have improved the disease trajectory [15]. However, despite restoration of the airway surface fluid with these personalised therapies, CFTR modulators do not significantly change bacterial diversity or *P. aeruginosa* load in the lung [16] and there is a clinical requirement for ongoing *P. aeruginosa* surveillance in the CF airways. Therefore, there is a need to develop accurate non-invasive methods to detect and quantify *P. aeruginosa* in CF without the need to use invasive techniques such as bronchoalveolar lavage.

Focusing on AQ detection in saliva for the early diagnosis of *P. aeruginosa* is biologically justified. The oral cavity is a potential reservoir for *P. aeruginosa* for initial colonisation which may

Table 1
The sensitivity, specificity, positive predictive value and negative predictive value of saliva AQs with *P. aeruginosa* PCR measured in sputum ($N = 75$).

	P. aeruginosa PCR +	*P. aeruginosa* PCR −	Total	
Saliva AQ +	35	0	35	PPV (95%CI); 100% (90.0;100.0)
Saliva AQ −	35	5	5	NPV (95%CI); 12.5%(4.2;26.8)
Total	70	5	75	McNemar's chi2; 35.00 ($p < 0.001$)
	Sensitivity (95%CI)	Specificity (95%CI)	ROC area (95%CI)	
	50% (37.8;62.2)	100% (47.8;100.0)	0.75 (0.70;0.81)	

AQ, 2-alkyl-4-quinolones; +, positive; −, negative; PPV, positive predicted value; NPV, negative predictive value; ROC area, Receiving operator characteristic area curve (Sensitivity + Specificity/2)
Out of the original cohort of 89 participants, 75 sputum samples were available for qPCR analysis.

Table 2
Correlations of AQs detected in saliva with AQs detected in spontaneous sputum and *P. aeruginosa* measured by PCR.

	Spearman's correlations: *r* (p-value)				
AQ in saliva	*N* (%)	AQ in sputum[a]	95%CI	*P. aeruginosa* PCR	95%CI
HHQ	34 (38)	0.65 (<0.001)	0.51;0.79	0.48 (<0.001)	0.30;0.65
NHQ	32 (36)	0.52 (<0.001)	0.34;0.69	0.37 (<0.001)	0.18;0.56
HQNO	26 (29)	0.62 (<0.001)	0.49;0.74	0.44 (<0.001)	0.26;0.62
NQNO	30 (34)	0.71 (<0.001)	0.61;0.82	0.44 (<0.001)	0.26;0.63
C7-PQS	10 (11)	0.45 (<0.001)	0.28;0.62	0.20 (0.081)	0.01;0.40
C9-PQS	9 (10)	0.43 (<0.001)	0.26;0.60	0.20 (0.091)	0.02;0.37

AQ, 2-alkyl-4 quinolones; 95%CI, 95% confidence intervals; n, number; HHQ, 2-heptyl-4-hydroxyquinoline; NHQ, 2-nonyl-4-hydroxyquinoline; C7-PQS, 2-heptyl-3-hydroxy-4(1*H*)-quinolone; C9-PQS, 2-nonyl-3-hydroxy-4(1*H*)-quinolone; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; NQNO, 2-nonyl-4-hydroxyquinoline-*N*-oxide, N; number of samples with AQ detected out of a total of 89 samples [a]; n = 77; [b]; n = 75, where n is the number of samples with data available for matched analysis.

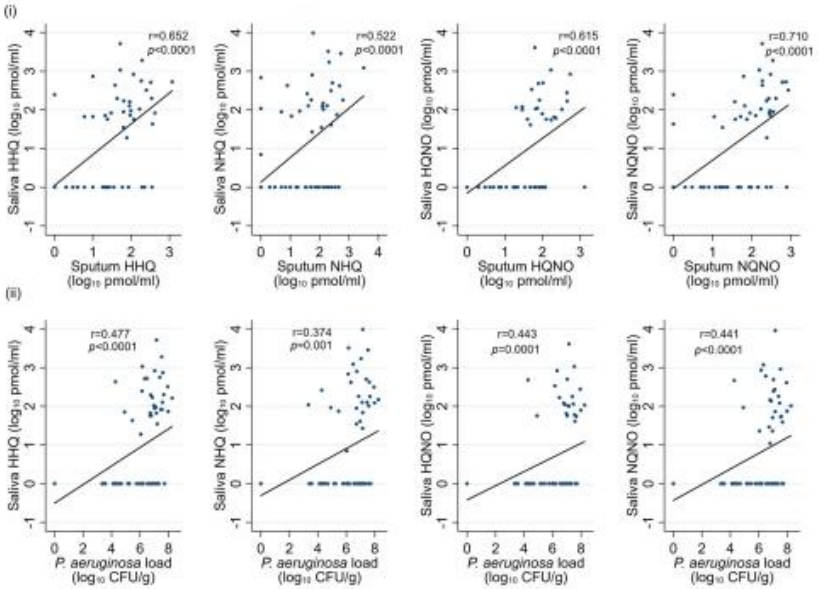

Fig. 1. Spearman correlations between salivary concentrations of four AQs and (i) spontaneous sputum AQs concentrations and (ii) and *P. aeruginosa* load measured in sputum using PCR.

precede chronic lung infection [17] and this is supported by data showing similarities between the oral and lung microbiota [18]. In addition, AQs upregulate virulence factors known to be important in establishing early infection and AQs have been detected in the systemic circulation of people with CF during early *P. aeruginosa* infection [19].

This study has some limitations that should be considered when interpreting these data. Firstly, this study is a proof of concept pilot study. These findings need to be validated, including with a control population of people with CF who do not have pulmonary *P. aeruginosa*, to accurately determine its potential use in the clinical setting. Secondly, participants rinsed their mouths with tap water prior to saliva sample collection and this may have adversely impacted the sensitivity of the analysis or led to false positive results due to potential water contamination. From our results, we cannot determine if the AQs detected in the saliva originate from the upper airway sources or from oral contamination from expectorated sputum from lower airways colonisation. AQs are also produced by a few other related bacterial species, including *Burkholderia cepacia* complex [7,10,20]. Participants known to have previously isolated Burkholderia cepacia complex were excluded from the study to minimise this risk. In addition, chronic *P. aeruginosa* infections in the CF lung can give rise to QS deficient mutants (such as LasR mutants) [21]. However, there has been no recorded evidence of AQ mutants in this setting to date [22,23].

One limitation to the potential use of salivary AQs as an early non-invasive diagnostic assay for *P. aeruginosa* infection is that the sensitivity is only 44%. However, this is simply a proof of principle study demonstrating for the first time that salivary AQs can measure *P. aeruginosa* infection. Further studies refining the use of salivary AQs in the early diagnosis of *P. aeruginosa* infection can aim to increase the sensitivity of this approach; possibilities include the collection of three samples and either avoidance of rinsing the mouth prior to saliva collection or using de-ionised sterile water. In addition, refinement of the liquid chromatography-tandem mass spectrometry technology is needed to increase the sensitivity of detecting salivary AQs. Finally, further prospective studies with regular longitudinal sampling of salivary samples are needed to determine the variability of AQs in vivo and ensure both the accuracy and generalisability of these findings to the wider CF population as a whole.

In summary, *P. aeruginosa* QS signal molecules can be detected in the saliva obtained from adults with CF and chronic pulmonary *P. aeruginosa* infection. Salivary AQ levels correlated significantly with both the concentrations of AQs and *P. aeruginosa* in the sputum. However, further refinement of the methodologies is needed to improve the sensitivity of detecting AQs in saliva to be clinically useful as non-invasive biomarkers for pulmonary *P. aeruginosa* in the future.

CRediT author statement

The study was designed by KW/HLB/AWF/MC. Conceptualization, data curation and funding acquisition was performed by HLB/AWF/MC/PW. The investigation, methodology, software supervision, validation, visualization, writing-original draft and writing-review and editing was performed by KW, HLB and AWF. The PCR data analysis was performed by MZ and the AQ analysis was performed by NH. All authors contributed to data interpretation, data presentation and writing of the manuscript. All authors approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2021.08.030.

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MICROBIOLOGY SOCIETY

2-Alkyl-4-quinolone quorum sensing molecules are biomarkers for culture-independent *Pseudomonas aeruginosa* burden in adults with cystic fibrosis

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Abstract

Introduction. *Pseudomonas aeruginosa* produces quorum sensing signalling molecules including 2-alkyl-4-quinolones (AQs), which regulate virulence factor production in the cystic fibrosis (CF) airways.

Hypothesis/Gap statement. Culture can lead to condition-dependent artefacts which may limit the potential insights and applications of AQs as minimally-invasive biomarkers of bacterial load.

Aim. We aimed to use culture-independent methods to explore the correlations between AQ levels and live *P. aeruginosa* load in adults with CF.

Methodology. Seventy-five sputum samples at clinical stability and 48 paired sputum samples obtained at the beginning and end of IV antibiotics for a pulmonary exacerbation in adults with CF were processed using a viable cell separation technique followed by quantitative *P. aeruginosa* polymerase chain reaction (qPCR). Live *P. aeruginosa* qPCR load was compared with the concentrations of three AQs (HHQ, NHQ and HQNO) detected in sputum, plasma and urine.

Results. At clinical stability and the beginning of IV antibiotics for pulmonary exacerbation, HHQ, NHQ and HQNO measured in sputum, plasma and urine were consistently positively correlated with live *P. aeruginosa* qPCR load in sputum, compared to culture. Following systemic antibiotics live *P. aeruginosa* qPCR load decreased significantly ($P<0.001$) and was correlated with a reduction in plasma NHQ (plasma: $r=0.463$, $P=0.003$).

Conclusion. In adults with CF, AQ concentrations correlated more strongly with live *P. aeruginosa* bacterial load measured by qPCR compared to traditional culture. Prospective studies are required to assess the potential of systemic AQs as biomarkers of *P. aeruginosa* bacterial burden.

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Keywords: alkyl-quinolones; cystic fibrosis; PMA-qPCR; *Pseudomonas aeruginosa*; quorum sensing molecules.
Abbreviations: AQs, 2-alkyl-4-quinolones; CF, cystic fibrosis; DNA, deoxyribonucleic acid; HHQ, 2-heptyl-4-hydroxyquinoline; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; IV, intravenous; LLOQ, lowest limit of quantification; NHQ, 2-nonyl-4-hydroxyquinoline; PMA, propidium monoazide; qPCR, quantitative polymerase chain reaction.
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One supplementary table and three supplementary figures are available with the online version of this article.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium extremely well adapted to the airway niche in people with cystic fibrosis (CF). *P. aeruginosa* is a dominant pathogen in the CF lung and is associated with increased morbidity and mortality in this population [1]. Treatment and eradication of *P. aeruginosa* is a challenge as it is intrinsically resistant to many classes of antibiotics, produces a host of virulence factors, and forms impenetrable biofilms in the CF airways [2].

P. aeruginosa controls the production of virulence factors using cell-to-cell communication mechanisms known as quorum sensing (QS) [3]. One of the *P. aeruginosa* QS systems is based on the production and sensing of 2-alkyl-4-quinolones (AQs), which control virulence in a population dependent manner [4–7]. These AQs are only produced by *P. aeruginosa* and several closely related species [8].

Our previous studies have shown that several AQs are detectable in the sputum, plasma and urine of adults with CF and chronic pulmonary *P. aeruginosa* [9, 10]. Concentrations of two AQs, HHQ (2-heptyl-4-hydroxyquinoline) and HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) were most strongly associated with *P. aeruginosa* load measured by routine sputum culture at clinical stability [10]. Following intravenous anti-pseudomonal antibiotics for a pulmonary exacerbation, the concentrations of NHQ (2-nonyl-4-hydroxyquinoline) and HHQ in the plasma declined significantly, but there was no change in *P. aeruginosa* load, measured by routine culture [9].

In this study, we compared *P. aeruginosa* load measured historically by culture with live *P. aeruginosa* load quantified by polymerase chain reaction (qPCR). By using a photoreactive dye, propidium monoazide (PMA), we were able to differentiate between viable and compromised *P. aeruginosa* cells. With this modification, only viable (live *P. aeruginosa*) cells are amplified and quantified during the qPCR process [11]. We aimed to explore whether three previously defined AQs; HHQ, NHQ, and HQNO correlated more strongly with culture-independent measures of live *P. aeruginosa* load compared to a culture-dependent method. We tested correlations of these three AQs detected in sputum, plasma, and urine with *P. aeruginosa* in the sputum at stability and in response to treatment for a pulmonary exacerbation.

METHODS

Participants and study design

Spontaneous sputum samples were obtained and stored from adults with CF who had previously participated in two AQ biomarker studies, details of which have been previously published [9, 10]. In the original studies, baseline demographic data were collected, and matched duplicate sputum plugs were frozen at −80 °C for future studies. In summary, spontaneous sputum, plasma and urine samples were obtained from 75 adults with CF at clinical stability and from 48 adults at the start and end of intravenous (IV) antibiotic treatment for pulmonary exacerbation, according to Rosenfeld criteria [12]. Adults aged 16 to 60 were recruited from two UK adult CF centres, who were known to have previously isolated *P. aeruginosa* from respiratory samples obtained during routine clinical practice. Spontaneous sputum, 8 ml venous blood and 25 ml urine samples were obtained at stability or within 72 h of the start and end of IV antibiotic therapy [9].

PMA-based qPCR analysis

Prior to DNA extraction, sputum samples were pre-treated with propidium monoazide (PMA) to penetrate dead/compromised bacterial cells and bind to DNA as described previously by Rogers *et al.* [13]. PMA (20 mM in water; Biotium, USA) was added to the samples to a final concentration of 50 µM followed by incubation in the dark on a rotating shaker for 30 min. This allowed the PMA molecules to penetrate only dead/compromised bacterial cells as they were impermeable to intact cell membrane. The PMA molecules were then fixed to DNA by 15 min exposure to LED blue light (IB-Applied Science, Spain). Upon exposure to PMA and light, permanent DNA modification was achieved by formation of stable covalent nitrogen-carbon bond, which prevented amplification (of DNA from dead/compromised cells) during qPCR analysis. Cells were pelleted at 10000 ***g*** for 5 min prior to DNA extraction.

DNA extraction was conducted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich Co. Ltd., Dorset, UK) with the following modifications. Samples were initially mixed with lysozyme (200 µl; 45 mg ml^{-1}, Sigma-Aldrich Co. Ltd., Dorset, UK) suspended in Gram-Positive Lysis Solution (included in the kit) followed by insertion of glass beads. Cell disruption was then achieved by agitation in a Fastprep-24 Instrument (MP Biomedicals Europe, Illkirch, France) at 6.5 m s^{-1} for 60 s twice and subsequently incubated at 37 °C for 30 min. Further steps remained unchanged, and the DNA was resuspended in 50 µl of elution solution (included in the kit). DNA concentrations were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK). *P. aeruginosa*-specific qPCR assay was performed as described previously [14]. The lower limit of quantification (LLOQ) for *P. aeruginosa* in this assay was 100 c.f.u. g^{-1} of sputum aliquot. Quantitative values were generated by Rotor Gene Q-series software (Qiagen, Crawley, UK) and expressed in c.f.u. g^{-1} of sputum aliquot.

Sample processing for quantitative culture and AQ analyses

All sample processing for quantitative culture, differential cell counts and AQ analyses was performed in the initial AQ biomarker studies as previously described [9, 10]. Prepared clinical samples for AQ analyses were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Ortori *et al.* [15]. Further information regarding the methodology of AQ analyses and the calculated lower limits of quantification (LLOQ) are provided in the online supplementary material. To reduce multiple hypotheses

Table 1. Baseline demographics of participants at clinical stability (*n*=75) and exacerbation (*n*=48)

Variable	Baseline
Clinical stability cohort	*n*=75
Age in years: median (range)	38.6 (17.8 to 61.5)
Gender, males (%)	49 (65.3)
FEV_1 % predicted: mean (SD)	55 (±18)
Absolute FEV_1 in L: mean (SD)	2.0 (±0.8)
BMI: mean (SD)	22.9 (±3.3)
P. aeruginosa status by culture at baseline: n (%)	
Never	0 (0)
Free	1 (1.3)
Intermittent	2 (2.7)
Chronic	72 (96)
Exacerbation cohort	*n*=48
Age in years: median (range)	27.5 (17 to 59)
Gender, males (%)	27 (56.3)
FEV_1 % predicted: mean (SD)*	46.6 (±16)
Absolute FEV_1 in L: mean (SD)*	1.7 (±0.7)
BMI: mean (SD)	21.9 (±3.9)
Diagnostic microbiology results: n (%)	
P. aeruginosa isolated only:	33 (68.8)
P. aeruginosa co-infection with:	11 (22.9)
MSSA	4 (36.4)
MRSA	1 (9.1)
Haemophilus influenza and *MSSA*	1 (9.1)
Aspergillus fumigatus and *MSSA*	1 (9.1)
Candida albicans	4 (36.4)
No *P. aeruginosa* isolated:	4 (8.3)
MSSA	1 (25)
MRSA	2 (50)
Respiratory commensals	1 (25)

*Spirometry at exacerbation. *P. aeruginosa* status of participants defined by *Leeds criteria* [22].
n, number of participants with data available; SD, standard deviation.

testing, only the three AQs which were most strongly associated with *P. aeruginosa* burden from the previous study were analysed [9, 10]: HHQ (2-heptyl-4-hydroxyquinoline), NHQ (2-nonyl-4-hydroxyquinoline) and HQNO (2-heptyl -4-hydroxyquinoline-*N*-oxide).

Statistical analysis

Any value that was under the LLOQ was designated as undetected. AQ levels and *P. aeruginosa* qPCR loads were analysed after the addition of one to each value followed by $\log_{10}$ transformation to scale the data for interpretation. For analysis of absolute difference between two time points (at the beginning and end of IV antibiotics for pulmonary exacerbation), no log transformation was performed on the values. When paired t-tests were used, normality of the data distribution was assessed both visually and using Shapiro-Wilk Test of Normality.

Comparisons of FEV_1 measurements, sputum neutrophil concentrations, *P. aeruginosa* load measured by qPCR and *P. aeruginosa* load measured by culture at the beginning and end of IV antibiotics were analysed using paired t-tests. Quantitative loads of *P. aeruginosa* from culture and qPCR were assessed using Spearman's rank correlations, as well as sputum *P. aeruginosa* qPCR data with HHQ, NHQ and HQNO measured in blood, sputum and urine. Correlation coefficients of *P. aeruginosa* measured by culture and qPCR were transformed from r values to z scores using Fisher Z transformation to identify whether the z test statistic was outside the critical value, defined using two-tailed alpha 0.05 [16]. Post-IV antibiotics changes in absolute FEV_1 measurements, *P. aeruginosa* load (qPCR and culture) and AQ levels were calculated using the values at the end of IV antibiotics minus the values at the beginning of IV antibiotics. All statistical analyses were performed using Stata SE15 statistical software (Texas, USA).

RESULTS

Sputum aliquots from 75 adults at clinical stability and 48 adults at the start and end of IV antibiotic treatment were available and subsequently thawed and processed for qPCR analysis. Based on the 16S rRNA gene sequencing data we obtained, *Burkholderia* sp. and *Acinetobacter* sp. were not present in any of the samples.

Baseline demographics of participants included in the analysis at clinical stability and exacerbation are summarised in Table 1.

Individual AQ molecules are associated with live *P. aeruginosa* qPCR load at clinical stability compared to *P. aeruginosa* culture load

At clinical stability, there was no correlation between live *P. aeruginosa* load measured by qPCR and the load detected by culture (r=0.174, *P*=0.166, Fig. S1, available in the online version of this article). All of the three AQs (HHQ, NHQ, HQNO) consistently correlated with live *P. aeruginosa* load detected by qPCR in all sample types (sputum, plasma and urine, Fig. 1). The strongest relationship with live *P. aeruginosa* qPCR load was observed with plasma HHQ (r=0.550, r^2=0.30, *P*<0.001, Fig. 1).

In contrast, *P. aeruginosa* quantitative load measured by culture only correlated with HHQ and NHQ measured in sputum (r=0.381, *P*=0.002; r=0.296, *P*=0.017) and urine (r=0.264, *P*=0.034; r=0.322, *P*=0.012), respectively (Table 2). No correlations were observed with any AQs measured in plasma. Comparison of the correlation coefficients of *P.*

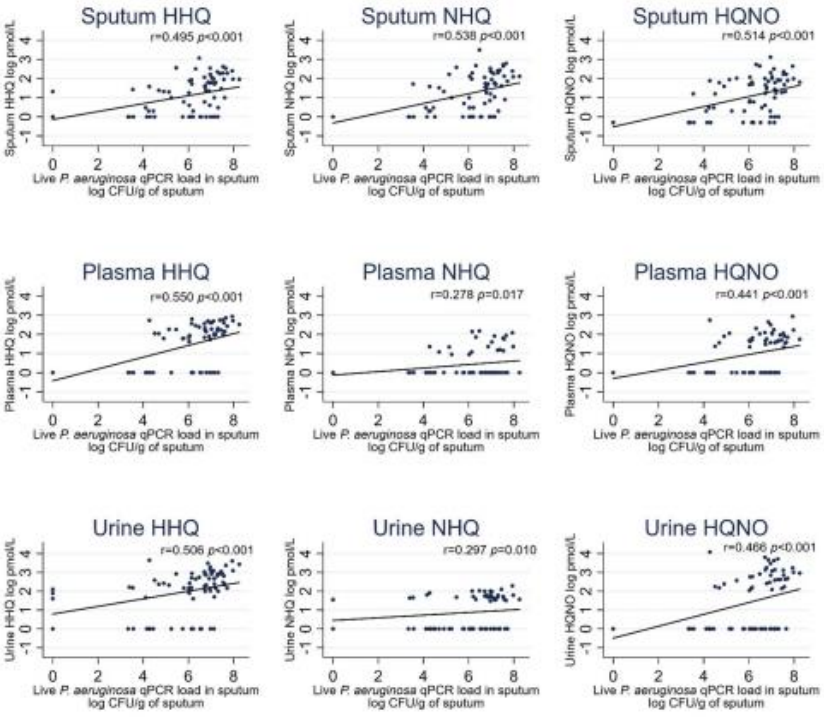

Fig. 1. Correlations of HHQ, NHQ and HQNO detected in sputum, plasma and urine with live *P. aeruginosa* load by qPCR in sputum during clinical stability. Median (interquartile range) for sputum live *P. aeruginosa* load by qPCR was 3.3×10^{4} c.f.u.g^{-1} (1.4×10^{3}–1.4×10^{7} c.f.u.g^{-1}) of sputum. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline. HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide; Sputum, *n*=75; plasma, *n*=74; urine, *n*=75, where n is the number of participants with data available for statistical analysis. Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol l^{-1}), NHQ (10, 10 pmol l^{-1}) and HQNO (30, 30 pmol l^{-1}). No LLOQ was defined for sputum AQs. The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells g^{-1} of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation.

aeruginosa load measured by qPCR against culture demonstrated significantly higher correlations on qPCR compared to culture in sputum NHQ and HQNO, plasma HHQ and HQNO, and urine HHQ and HQNO (Table 2).

Clinical and microbiological changes in response to treatment for a pulmonary exacerbation

There was a significant correlation between live *P. aeruginosa* load measured by qPCR and the load detected by culture at the beginning of IV antibiotics for pulmonary exacerbation (r=0.520, *P*=<0.001). Correlation was not observed when IV antibiotics were stopped (r=0.258, *P*=0.083, Fig. S1).

Following treatment for a pulmonary exacerbation, the mean absolute FEV_1 increased from 1.68 L (SD ±0.68) at the beginning of IV antibiotics to 1.96 L (SD ±0.82) at the end of IV antibiotics, (*P*=<0.0001). Mean percent (%) predicted

Table 2. Correlations of HHQ, NHQ and HQNO in sputum, plasma and urine with live *P. aeruginosa* loads measured by qPCR at clinical stability compared to historical quantitative *P. aeruginosa* load by culture

		Sputum	Plasma	Urine
	AQ	Spearman's Correlation Coefficient for qPCR, r (*P*-value)+		
qPCR	HHQ	0.495 (<0.001)*	0.550 (<0.001)*	0.506 (<0.001)*
	NHQ	0.538 (<0.001)*	0.278 (0.017)*	0.297 (0.010)*
	HQNO	0.514 (<0.001)*	0.441 (<0.001)*	0.466 (<0.001)*
		Spearman's Correlation Coefficient for Culture, r (*P*-value)β		
Culture	HHQ	0.381 (0.002)*	0.232 (0.065)	0.264 (0.034)*
	NHQ	0.296 (0.017)*	0.121 (0.342)	0.311 (0.012)*
	HQNO	0.203 (0.104)	0.169 (0.183)	0.197 (0.115)
		Fisher Z transformation qPCR vs Culture (*P*-value)†		
qPCR vs culture	HHQ	0.818 (0.207)	2.187 (0.014)*	1.661 (0.048)*
	NHQ	1.708 (0.044)*	0.940 (0.174)	−0.088 (0.465)
	HQNO	2.090 (0.018)*	1.733 (0.042)*	1.760 (0.039)*

Median (interquartile range) for sputum live *P. aeruginosa* load by qPCR was 3.3×10^{6} c.f.u.g^{-1} (1.4×10^{6}–1.4×10^{7} c.f.u.g^{-1}) of sputum. Median (interquartile range) for sputum *P. aeruginosa* load by culture was 1.2×10^{7} c.f.u.g^{-1} (2.8×10^{6}–3.28×10^{7} c.f.u.g^{-1}) of sputum. Analysis with qPCR: Sputum, *n*=75; plasma, *n*=74; urine, *n*=75. Analysis with Culture: Sputum, *n*=65; plasma, *n*=64; urine, *n*=65 where *n* is the number of patients with samples available for analysis. Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol l^{-1}), NHQ (10, 10 pmol l^{-1}) and HQNO (30, 30 pmol l^{-1}). No LLOQ was defined for sputum AQs. The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells g^{-1} of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation.
*Significant at *P*<0.05.
†*Z* test statistic for difference between spearman rho values.
AQ, 2-Alkyl-4-quinolone; HHQ, 2-heptyl-4-hydroxyquinoline; NHQ, 2- nonyl-4-hydroxyquinoline; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide.

FEV_1 increased from 46.6% (SD ±16) to 52.4% (SD ±17.8), (*P*=<0.0001, Table S1).

The mean number of neutrophils detected in sputum significantly reduced following IV antibiotic treatment for a pulmonary exacerbation, from 7.15 log cells g^{-1} (SD ±0.37) to 6.9 log cells g^{-1} (SD ±0.38), (*P*<0.001). Mean *P. aeruginosa* load detected by culture showed no significant change between the beginning of IV antibiotics; 6.59 $\log_{10}$CFU g^{-1} (SD ±1.83) and the end of IV antibiotics; 6.67 $\log_{10}$CFU g^{-1} (SD ±1.82), (*P*=0.84). Mean live *P. aeruginosa* load measured by qPCR showed a significant reduction from 6.40 $\log_{10}$CFU g^{-1} (SD ±1.52) at the beginning of IV antibiotics, to 5.71 $\log_{10}$CFU g^{-1} (SD ±1.97) at the end of IV antibiotics (*P*=<0.001, Table S1).

Changes in absolute FEV_1 measurements and live *P. aeruginosa* measured by qPCR were negatively correlated (r=−0.415, *P*=0.004, Fig. S2). There was no correlation between changes in absolute FEV_1 and *P. aeruginosa* measured by culture (r=0.115, *P*=0.473, Fig. S2).

HHQ and NHQ concentrations are associated with live *P. aeruginosa* PCR load before and after pulmonary exacerbation

At the start of pulmonary exacerbation HHQ, NHQ and HQNO concentrations measured in sputum, plasma and urine showed positive correlations with live *P. aeruginosa* load measured by qPCR (*P*=<0.001, Table 3). The strongest correlation was with sputum NHQ (r=0.736, r^2=0.54, *P*<0.001). *P. aeruginosa* load measured by qPCR are consistent with *P. aeruginosa* load measured previously by culture with the exception of urinary HQNO. Correlations between HHQ and NHQ measured in the urine were significantly stronger with *P. aeruginosa* qPCR when compared to culture (Table 3).

At the end of antibiotic treatment, HHQ levels were positively correlated with live *P. aeruginosa* load measured by qPCR in all sample types (sputum r=0.527, *P*<0.001; plasma r=0.430, *P*<0.001; urine r=0.509, *P*<0.001, Table 4). Significant correlations of live *P. aeruginosa* load measured by qPCR were observed in sputum NHQ (r=0.554, *P*=0.001), sputum HQNO (r=0.583 *p*<0.001) and urine HQNO (r=0.411 *P*=0.004) but not in plasma (Table 4). These findings are consistent with previous *P. aeruginosa* loads measured in culture with the exception of plasma HHQ and urine HQNO which showed significant correlations with qPCR load but not culture.

Changes in live *P. aeruginosa* load measured using qPCR positively correlated with changes in NHQ signal concentration in plasma

Further analyses were performed to investigate the relationship between absolute changes of live *P. aeruginosa* measured by qPCR with changes in HHQ, NHQ and HQNO concentrations following IV antibiotics for a pulmonary exacerbation. A positive correlation was observed between change in plasma NHQ following systemic antibiotics and changes in live *P. aeruginosa* loads measured by qPCR in sputum (r=0.422, *P*=0.003, Table 4, Fig. S3). No other significant correlations were found between differences in absolute live *P. aeruginosa*

Table 3. Correlations of HHQ, NHQ and HQNO concentrations in sputum, plasma and urine with sputum live *P. aeruginosa* load measured using qPCR at the start and end of IV-antibiotic treatment for a pulmonary exacerbation compared to historical culture data

	Start of IV antibiotics†			End of IV antibiotics†		
	qPCR	Culture	qPCR vs culture	qPCR	Culture	qPCR vs culture
AQ	Spearman's Correlation Coefficient, r (*P*-value)		Z (*P*-value)‡	Spearman's Correlation Coefficient, r (*P*-value)		Z (*P*-value)‡
HHQ						
Sputum	0.626 (<0.001)*	0.522 (<0.001)*	0.725 (0.234)	0.527 (<0.001)*	0.345 (0.019)*	1.059 (0.145)
Plasma	0.524 (<0.001)*	0.546 (<0.001)*	−0.141 (0.444)	0.430 (0.003)*	0.121 (0.430)	1.570 (0.058)
Urine	0.607 (<0.001)*	0.306 (0.041)*	1.810 (0.035)*	0.509 (<0.001)*	0.295 (0.047)*	1.207 (0.114)
NHQ						
Sputum	0.736 (<0.001)*	0.532 (<0.001)*	1.627 (0.052)	0.554 (<0.001)*	0.297 (0.045)*	1.486 (0.069)
Plasma	0.452 (0.001)*	0.314 (0.038)*	0.746 (0.228)	0.234 (0.113)	0.103 (0.500)	0.625 (0.228)
Urine	0.636 (<0.001)*	0.350 (0.019)*	1.799 (0.036)*	0.248 (0.090)	0.216 (0.150)	0.157 (0.438)
HQNO						
Sputum	0.600 (<0.001)*	0.431 (0.003)*	1.082 (0.140)	0.583 (<0.001)*	0.294 (0.047)*	1.707 (0.044)*
Plasma	0.577 (<0.001)*	0.479 (0.001)*	0.630 (0.264)	0.206 (0.166)	0.115 (0.453)	0.432 (0.333)
Urine	0.524 (<0.001)*	0.273 (0.070)	1.404 (0.273)	0.411 (0.004)*	0.222 (0.138)	0.988 (0.162)

†Median (interquartile range) for sputum live *P. aeruginosa* load was 8×10^{6} c.f.u.g^{-1} (3×10^{5}–2×10^{8} c.f.u.g^{-1}) at pre-antibiotics and 2×10^{5} c.f.u.g^{-1} (1×10^{4}–7×10^{7} c.f.u.g^{-1}) at post-antibiotics. ‡AQ: 2-Alkyl-4-quinolone, HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline.HQNO: 2-heptyl-4-hydroxyquinoline-*N*-oxide. qPCR: Sputum, *n*=48; plasma, *n*=47; urine, *n*=48. Culture: Sputum, *n*=45; plasma, *n*=44; urine, *n*=45, (where n is the number of patients with samples available for analysis). Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol l^{-1}), NHQ (10, 10 pmol l^{-1}) and HQNO (30, 30 pmol l^{-1}). No LLOQ was defined for sputum AQs. The LLOQ for *P. aeruginosa* measured by qPCR was 100 cells g^{-1} of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation. ‡¶ Z test statistic for difference between spearman rho values. *Significant comparison at *P*<0.05

qPCR load and changes in NHQ, HHQ or HQNO concentrations (NHQ in sputum and urine; HHQ and HQNO in sputum, plasma and urine). There were no correlations with changes in *P. aeruginosa* measured by culture and changes with any AQ in all sample types (Table 4).

DISCUSSION

This is the first study to explore systemic measurements of AQ quorum sensing molecules as biomarkers of live *P. aeruginosa* load measured by qPCR in the sputum in adults with CF. Overall, there were stronger correlations between AQ concentrations and qPCR measures of bacterial load compared to previous culture methodology which is likely to be less standardised therefore contributing to higher levels of measurement error. In contrast to *P. aeruginosa* load measured by culture at clinical stability, qPCR *P. aeruginosa* load showed consistent correlations with HHQ, NHQ and HQNO measured in sputum, plasma and urine. At the beginning of IV antibiotics for pulmonary exacerbation, HHQ and NHQ in sputum, plasma and urine were positively correlated with *P. aeruginosa* load measured by both qPCR and culture. In addition, absolute change of sputum *P. aeruginosa* load measured by qPCR were reflected by an absolute change in plasma NHQ between the start and end of IV antibiotics for pulmonary exacerbation.

We found no consistent correlations between culture and qPCR measures of *P. aeruginosa* load during stability or following IV antibiotic treatment, although correlations were observed at the beginning of IV antibiotics for a pulmonary exacerbation. The *P. aeruginosa* load measured by qPCR reduced significantly after antibiotic treatment whilst there was no significant difference using culture. It is possible that qPCR assays may be more sensitive at detecting *P. aeruginosa* compared to culture [17]. PMA-qPCR may more accurately quantify viable uncultivable bacterial cells which are metabolically dormant and therefore not detected by culture, as suggested by Deschaght *et al.* [18] demonstrating the potential strengths of PMA-qPCR compared to culture. This theory supports our observations that the correlation between qPCR quantification of *P. aeruginosa* load and AQs were stronger compared to culture. However, we found higher levels of *P. aeruginosa* detected by culture compared to qPCR quantification. It is possible that the freeze-thaw cycle may have reduced the viable *P. aeruginosa* in the sputum samples that underwent qPCR analysis. Alternatively, quantification by culture may be subject to individual operator error and inaccuracy [19].

In our study, only *P. aeruginosa* load measured by qPCR was correlated with improvements in absolute FEV_1 following treatment for a pulmonary exacerbation, and not culture. There are conflicting data in the literature regarding the relationship between culture and culture-independent quantitative *P. aeruginosa* load measurements with CF related outcomes [18, 20–22]. For example, McLaughlin *et al.* showed no correlation between

Table 4. Correlations between changes in sputum, plasma and urine AQ concentrations with live *P. aeruginosa* load changes after IV antibiotic treatment

Changes post-IV antibiotics	qPCR	Culture	qPCR vs culture
AQ	Spearman's Correlation Coefficient, r (*P*-value)		Z (*P*-value)†
HHQ			
Sputum	0.252 (0.083)	0.266 (0.085)	−0.065 (0.474)
Plasma	0.276 (0.061)	0.285 (0.067)	−0.047 (0.481)
Urine	0.182 (0.215)	−0.042 (0.787)	1.044 (0.148)
NHQ			
Sputum	0.237 (0.104)	0.202 (0.193)	0.169 (0.433)
Plasma	0.422 (0.003)*	0.230 (0.143)	0.917 (0.180)
Urine	0.274 (0.059)	0.082 (0.602)	0.982 (0.163)
HQNO			
Sputum	0.230 (0.115)	0.148 (0.345)	−0.001 (0.499)
Plasma	0.125 (0.404)	0.125 (0.431)	0.395 (0.346)
Urine	0.205 (0.163)	−0.109 (0.488)	1.457 (0.073)

Median (interquartile range) for sputum live P. aeruginosa qPCR load was 8 x 10^5 c.f.u. g^{-1} (5×10^4–3×10^7 c.f.u. g^{-1}) at pre-antibiotics and 2×10^6 c.f.u. g^{-1} (2×10^5–1×10^7 c.f.u. g^{-1}) post-antibiotics. Median (interquartile range) for culture *P. aeruginosa* load was 7 x 10^6 c.f.u. g^{-1} (8×10^5–6×10^7 c.f.u. g^{-1}) at pre-antibiotics and 1×10^7 c.f.u. g^{-1} (2×10^5–7×10^7 c.f.u. g^{-1}) post-antibiotics. HHQ: 2-heptyl-4-hydroxyquinoline; NHQ: 2-nonyl-4-hydroxyquinoline. HQNO: 2-heptyl-4-hydroxyquinoline-N-oxide. qPCR: Sputum, *n*=48; plasma, *n*=47; urine, *n*=48, Culture: Sputum, *n*=43; plasma, *n*=42; urine, *n*=48, (where n is the number of patients with samples available for analysis) Lowest limit of quantification (LLOQ) for plasma and urine AQs: HHQ (10, 20 pmol l^{-1}), NHQ (10, 10 pmol l^{-1}) and HQNO (30, 30 pmol l^{-1}). No LLOQ was defined for sputum AQs. The LLOQ for P. aeruginosa measured by qPCR was 100 cells g^{-1} of sputum. Values under LLOQ were designated a 0. All values had the addition of 1 prior to log transformation. †γ Z test statistic for difference between spearman rho values. *P value <0.05.

lung function improvement and a reduction of *P. aeruginosa* load measured by culture after pulmonary exacerbation [22]. However, other studies have shown a significant reduction in culture-dependent and culture-independent measures of *P. aeruginosa* load after treatment for pulmonary exacerbation, and these correlated significantly with lung function improvement [18, 23]. These variable observations are likely to be a result of different methodological approaches in measuring the quantitative load of *P. aeruginosa*. Here, we further demonstrate the dynamic relationship of AQs with longitudinal changes in quantitative *P. aeruginosa* load, which may offer novel opportunities to monitor bacterial burden during clinical stability and acute exacerbation in CF.

Strengths of this study include the large multicentre participant cohort and the pre-treatment of sputum with PMA which enabled only viable *P. aeruginosa* bacterial cells to be amplified and quantified. The sputum samples were collected and processed in line with conventional practice following a rigorous protocol, thereby reducing any variance in the signals generated by random error. In addition, the samples were homogenised and divided into aliquots before storage to avoid multiple freeze-thawing and loss of sample integrity. Furthermore, participants known to have previously isolated *Burkholderia cepacia complex* were excluded from the study as these organisms can also produce AQs [9, 10]. To limit multiple comparisons of different AQs, we assessed the three that were found to have the most promising biomarker potential from previous studies: HHQ, NHQ, HQNO [9, 10], however the type 1 error rate was not controlled.

There are a number of limitations in this study that should be considered when interpreting these data. Duplicate aliquots of sputum were frozen and thawed to perform *P. aeruginosa* qPCR analysis after the time-point of the quantification by culture. Therefore, this exposure to a longer period of frozen storage may limit direct comparisons between the burden of *P. aeruginosa* as measured by qPCR analysis and that quantified by culture colony forming units. This may account for the lack of correlation between the two different measures of P. aeruginosa load at clinical stability. *P. aeruginosa* bacterial load measured using qPCR accounted for up to 30% of the biomarker variability in AQs at clinical stability and 54% at pulmonary exacerbation. Variability in AQ concentrations that was not completely attributable to *P. aeruginosa* load may limit its potential clinical use as a marker of specific bacterial burden in clinical practice. This wide range of variability may be attributable to additional factors not measured in this study that could influence AQ concentration including oxygen gradients and nutrient availability in the lung environment resulting in heterogeneous conditions, which may lead to variability in sputum AQ concentrations [24]. Furthermore, the distribution, half-life and clearance of AQs in different sample types *in vivo* is not known. For example, the collection of urine samples used a random 'catch' method that may be associated with random error in the concentration of molecules being measured. To address the potential of urinary AQs as biomarkers, future studies should correct for differential

dilution with measurement of urinary creatinine, which may enhance the sensitivity to detect effects that were promising but non-significant in the limited longitudinal sample. Whilst analyses do not adjust for demographic or clinical factors, we reduced the effect of between-person variability in paired tests. Lastly, this was a retrospective study limited to adults with CF, further studies are needed to determine whether these findings are applicable to adults without CF and the wider CF population, including the paediatric CF population. Despite these limitations, there were associations between *P. aeruginosa* bacterial load in the sputum and systemic AQ concentrations in plasma and random urine samples, suggesting they may be useful in clinical practice as semi-quantitative, minimally-invasive biomarkers of infection.

Further investigation is needed to determine the relationship between AQs and bacterial burden in newly acquired *P. aeruginosa* pulmonary infection, to determine its potential as a screening tool for early infection. A minimally-invasive diagnostic test for *P. aeruginosa* would be especially useful in young children who cannot always expectorate sputum and may avoid the need for more invasive tests such as bronchoalveolar lavage. In addition, surrogate systemic measures of *P. aeruginosa* burden may prove increasingly useful in the era of highly effective CTFR modulator therapy where spontaneous sputum production is likely to decrease in the longer term [25].

In summary, dynamic changes in the bacterial load of *P. aeruginosa* objectively measured by qPCR were sensitive to the clinical course of exacerbation and intervention, which were not apparent from culture methods. AQs concentrations were more strongly correlated with culture independent methods of *P. aeruginosa* compared to culture dependent techniques. HHQ, NHQ and HQNO measured in sputum, plasma and urine were positively correlated with *P. aeruginosa* load measured by qPCR in sputum at pulmonary exacerbation and at clinical stability. In addition, changes in plasma NHQ reflect a reduction in *P. aeruginosa* after IV antibiotic treatment for pulmonary exacerbation, which has not been previously demonstrated. Further prospective investigation should seek to understand the sources of clinical variability in systemic AQ quorum sensing molecules, and confirm whether detection in plasma or urine can be reliably used as a minimally-invasive biomarker of *P. aeruginosa* load in people with CF.

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Author contributions
All included authors fulfil the criteria of authorship; N.M.Z. and K.W., are joint first authors for this manuscript. N.M.Z., K.W., H.L.B., A.F., M.C., I.S. and K.D.B., had substantial contributions to the study design. The qPCR analysis was performed by N.M.Z., the Q.S., analysis was performed by N.H. The statistical analysis was performed by N.M.Z., K.W. and I.S. All authors contributed to data interpretation, data presentation and drafting of the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest
M.C. and P.W., are partly funded by the National Biofilms Innovation Centre (NBIC) which is an Innovation and Knowledge Centre funded by the Biotechnology and Biological Sciences Research Council, InnovateUK and Hartree Centre (Award Number BB/R012415/1). ARS received research grant and consultancy fund from Vertex, and speaker honoraria and expenses from Teva and Novartis. D.A.B., A.R.S., D.L.F., A.F., M.C. and H.L.B., have a patent issued 'Alkyl-quinolones as biomarkers of *Pseudomonas aeruginosa* infection and uses thereof-PCT/GB2014/051458'. N.H. and P.W., has a patent WO/2014/184535 pending. N.M.Z., K.W., I.S., E.F.N., J.L.W., D.H., A.J.K. and K.D.B., declared that there are no conflict of interest.

Ethical statement
This study was approved by the Nottingham Research Ethics Committee 1(09/H0407/1).

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Porphyromonas pasteri and *Prevotella nanceiensis* in the sputum microbiota are associated with increased decline in lung function in individuals with cystic fibrosis

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Abstract

Although anaerobic bacteria exist in abundance in cystic fibrosis (CF) airways, their role in disease progression is poorly understood. We hypothesized that the presence and relative abundance of the most prevalent, live, anaerobic bacteria in sputum of adults with CF were associated with adverse clinical outcomes. This is the first study to prospectively investigate viable anaerobic bacteria present in the sputum microbiota and their relationship with long-term outcomes in adults with CF. We performed 16S rRNA analysis using a viability quantitative PCR technique on sputum samples obtained from a prospective cohort of 70 adults with CF and collected clinical data over an 8 year follow-up period. We examined the associations of the ten most abundant obligate anaerobic bacteria present in the sputum with annual rate of FEV_1 change. The presence of *Porphyromonas pasteri* and *Prevotella nanceiensis* were associated with a greater annual rate of FEV_1 change; −52.3 ml yr^{-1} (95% CI-87.7;−16.9), −67.9 ml yr^{-1} (95% CI-115.6;−20.1), respectively. Similarly, the relative abundance of these live organisms were associated with a greater annual rate of FEV_1 decline of −3.7 ml yr^{-1} (95% CI: −6.1 to −1.3, P=0.003) and −5.3 ml yr^{-1} (95% CI: −8.7 to −1.9, P=0.002) for each $\log_2$ increment of abundance, respectively. The presence and relative abundance of certain anaerobes in the sputum of adults with CF are associated with a greater rate of long-term lung function decline. The pathogenicity of anaerobic bacteria in the CF airways should be confirmed with further longitudinal prospective studies with a larger cohort of participants.

INTRODUCTION

Chronic pulmonary infection and recurrent pulmonary exacerbations are associated with both increased morbidity and mortality in cystic fibrosis (CF) [1]. Historically, conventional microbiological culture techniques have routinely isolated several distinct aerobic species, such as *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*, which have been extensively studied [2].

However, conventional culture techniques only identify a very small proportion of the bacteria present in the lung environment. In recent years, culture-independent techniques such as PCR and microbiome analysis have shown that the CF airways are complex polymicrobial environments and often contain anaerobic bacteria [3–5], which are not routinely cultured in clinical practice.

The CF airways contain mucus, which adheres to epithelial surfaces. Within these mucus plugs, are biofilms with steep oxygen gradients, which are considered to be hypoxic environments, providing a niche for anaerobic bacteria [6, 7]. However, it is

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Three supplementary tables are available with the online version of this article.

Table 1. Baseline clinical characteristics, summary of clinical data during the follow-up period and OTU abundance in each participant

Variable	Baseline (*n*=70)	
Age in years: median (range)	30.6 (17.8 to 61.5)	
Gender, males (%)	46 (65.1)	
FEV_1 % predicted: mean (SD)	58 (±20)	
Absolute FEV_1 in l: mean (SD)	2.2 (±0.9)	
BMI in kg m^{-2}: mean (SD)	22.9 (±3.4)	
P. aeruginosa status at baseline: *n* (%)		
Never	0 (0)	
Free	1 (1.4)	
Intermittent	1 (1.4)	
Chronic	68(97.1)	
Variable	**N (%)**	**Outcome**
Followup time* (years)	70 (100)	6.8 (6.2–8.1)
Number of lung transplantation	8 (11.4)	
Number of deaths	10 (14.2)	
Rate of lung function change per year†:		
Absolute FEV_1 (ml)	68 (97)	−53.2 (±71.4)
Percent predicted FEV_1 (%)	68 (97)	−1.4 (±1.9)
Variable per participant (*n*=70)	**Range**	**Mean (SD)**
Number of reads	9748–48 504	17 458.9 (±6306.1)
Number of OTUs	5–44	22.7 (±9.6)
Number of obligate anaerobe OTUs	0–17	6.3 (±4.4)
Total abundance, c.f.u. g^{-1} of sputum	5.0×10^{4}–3.9×10^{7}	3.5×10^{6} (6.0×10^{6})
Obligate anaerobe abundance, c.f.u. g^{-1} of sputum	0–7.6×10^{6}	6.1×10^{5} (1.3×10^{6})

n=number of participants with data available; SD=standard deviation, *P. aeruginosa* status of participants defined by Leeds criteria [17].
*Reported as median and interquartile range.
†Reported as mean and standard deviation.

currently not known whether these anaerobic bacteria play a pathophysiological role in lung damage, pulmonary exacerbations or long-term adverse outcomes in this patient population [6].

Sputum microbiome studies in CF have shown that the composition of bacterial communities remains relatively stable during exacerbations despite systemic antibiotic therapy [8, 9], and loss of species diversity is associated with reduced lung function [8, 10]. However, there are limited longitudinal data on the relationship between the sputum microbiome and long-term clinical outcomes [11]. Furthermore, whilst photoreactive dyes have been increasingly used to amplify and quantify only the bacterial cells with intact cell membranes in 16S rRNA sequencing [12], previous sputum microbiome studies in CF have not differentiated between viable and dead bacteria.

Using this viable cell technique, we investigated whether the presence and relative abundance of the ten most abundant, viable, obligate anaerobic bacterial species in CF sputum were associated with accelerated lung function decline in an 8 year follow up period.

METHODS

Participants and study design

We analysed sputum samples obtained adults with CF who had previously participated in a biomarker study (Research Ethics Committee; 09/H0407/1), the full details of which have been published [13]. In summary, participants were recruited at clinical

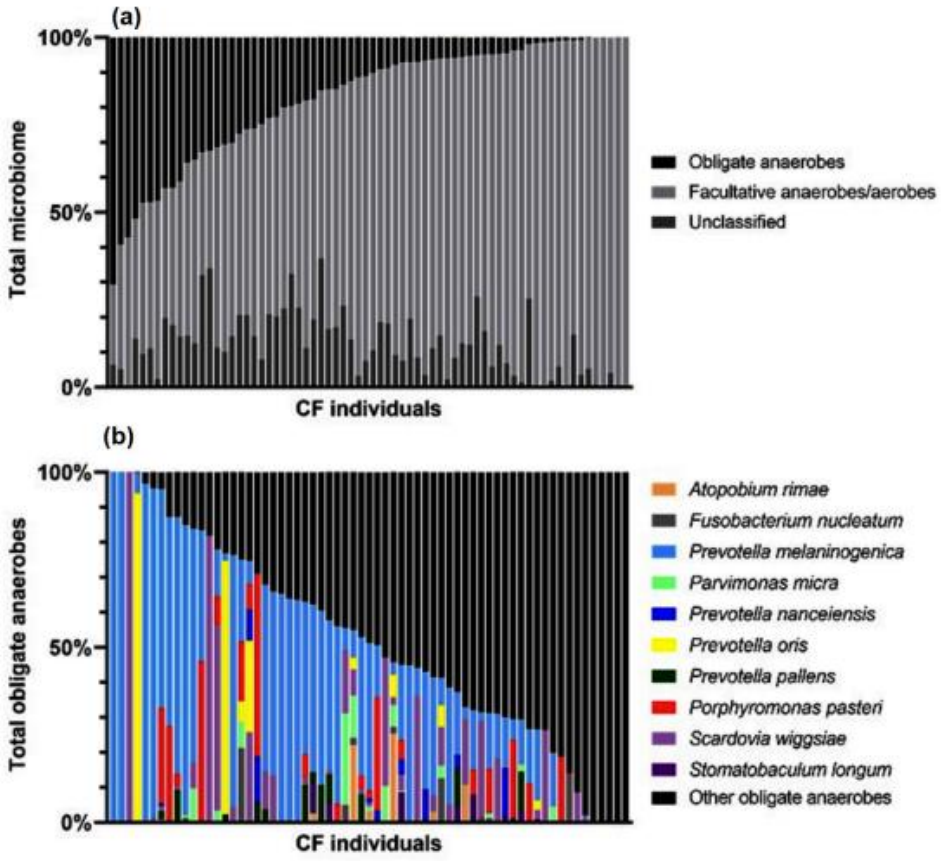

Fig. 1. Composition of (a) total obligate anaerobe OTUs within the viable CF total microbiome at clinical stability (n=70) and (b) individual OTUs included in the analysis within the total obligate anaerobe group (n=65).

stability from two UK adult CF centres between the years 2009 and 2011. Adults with CF were clinically stable at the study visit, having not experienced a pulmonary exacerbation requiring intravenous (IV) antibiotics in the preceding 4 weeks. Baseline demographic data were collected, and sputum plugs were stored for future microbiome analysis.

Clinical data

Lung function data were obtained from the UK CF registry [14] using the highest recorded forced expiratory volume in 1 s (FEV_1) of the preceding year. Annual data on lung function were collected from the participants from the year of recruitment to the end of the study period in 2017 or until year of death or lung transplantation.

Sample processing and microbiome analysis

Sputum plugs were harvested for microbiome analysis with an equal volume of 0.9% saline and stored in −80 °C freezers. Prior to DNA extraction, known weight of sputum aliquots (up to 0.2 g) were pre-treated with propidium monoazide (PMA). PMA is

Table 2. Effect size of the presence relative to the absence and relative abundance of the ten most abundant obligate anaerobic species on annual rate of FEV_1 (ml) decline, *N*=68

Anaerobic species	*N* (%)	Coefficient (β)±	95% CI±	Coefficient (β)*	95% CI*
Prevotella melaninogenica	50 (73.5)	24.7	−13.6; 63.0	0.4	−2.0; 2.8
Scardovia wiggsiae	15 (22.1)	−7.6	−43.0; 27.8	−1.8	−4.1; 0.5
Porphyromonas pasteri	9 (13.2)	−52.3	**−87.7; −16.9**	−3.7	**−6.1; −1.3**
Parvimonas micra	7 (10.3)	6.5	−34.7; 47.7	−0.7	−4.1; 2.7
Prevotella pallens	20 (29.4)	14.4	56.5; 27.8	−0.7	−3.8; 2.4
Stomatobaculum longum	9 (13.2)	11.8	−38.6; 62.2	−0.7	−4.4; 2.8
Fusobacterium nucleatum	8 (11.8)	35.4	14.3; 85.2	2.3	−1.7; 6.2
Prevotella nanceiensis	31 (45.6)	−67.9	**−115.6; −20.1**	−5.3	**−8.7; −1.9**
Prevotella oris	14 (20.6)	−11.2	−64.2; 41.8	−1.0	−4.4; 2.4
Atopobium rimae	9 (13.2)	−22.6	−78.6; 33.4	−2.0	−5.5; 1.5

N: number of bacteria present in sputum of participants, β: coefficient of annualized rate of FEV_1 decline (ml yr^{-1}) ±: Effect size of annual rate of FEV_1 decline if bacterial species is present relative to absent, *: log_2 relative abundance and annual rate of FEV_1 decline, 95% CI: confidence intervals. 95% CI in bold are considered significant after Bonferroni correction (*P*=0.005).

unable to penetrate intact cell membranes and therefore only binds to the DNA of cells with compromised cell membranes [15]. Photo-activation of PMA results in covalent DNA modification and damage. This subsequently prevents amplification via PCR. Non-PMA treated matching sputum samples were used as positive controls.

DNA extraction was conducted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, UK) according to the manufacturer's instructions, incorporated with additional enzymatic and physical disruption of samples. DNA was resuspended in 50 µl of Elution Solution and quantified using a Picodrop Microlitre Spectrophotometer (GRI, UK). Phosphate-buffered solution was used as negative controls in DNA extraction and library preparation for sequencing and no amplification was detected above the lowest limit of detection (10 c.f.u. μl^{-1}). Paired-end (300 bp) sequencing of 16S rRNA V3-V4 region for 75 DNA samples were then performed on the MiSeq platform (Illumina, USA). Sequence processing and analysis included quality filtering of reads, chimaera removal and assignment to Operational Taxonomic Units (OTUs) via the C9VLC pipeline and the QIIME software package (version 1.9.1, http://qiime.org/) (Eurofins, Germany).

Statistical analysis

Yearly change in lung function was predicted for each individual using linear regression models of CF registry-recorded FEV_1 values with their corresponding dates, and is reported as FEV_1 ml change per year (ml yr^{-1}).

The relative abundance of each OTU was initially expressed as a percentage (%) of the total number of reads in each individual. To allow comparison across the cohort, the % OTUs were transformed to c.f.u. (c.f.u. g^{-1}) of sputum by multiplying the % values with live total bacterial load in each sample accordingly, as informed by quantitative PCR (qPCR) [16]. The relative abundance (c.f.u. g^{-1} of sputum) was subsequently transformed after the addition of 1 (log_2) for statistical analyses. The ten most abundant OTUs were determined by ranking the relative abundance of the OTUs present in all participants (Table S1, available in the online version of this article). Only the taxonomic level of species were included in the analyses. Generalized linear regression models were used to analyse the presence or absence and relative abundance (log_2) on the annual rate of FEV_1 change. Model fit was assessed using Akaike and Bayesian information criterion and both the dependant variable (FEV_1 decline) and model residuals were assessed and confirmed to be normally distributed.

All analyses were performed in Stata SE15 statistical software (Texas, USA), a *P*-value<0.005 was considered statistically significant following Bonferroni correction for multiple comparison of ten OTUs.

RESULTS

Sputum samples and data were available for 70 (93.1%) of the 75 participants in the original study.

Sixty-eight (97.1%) of participants were chronically colonized with *P. aeruginosa* [17]. Baseline clinical characteristics and OTU abundance are summarized in Table 1.

A total of 1.2 million sequencing reads were obtained from the pooled sputum of each participant (n=70) and assigned to 212 OTUs. The assignment of 212 OTUs was determined by best-matching reference sequences to the most specific (lowest) taxonomic level. Within this dataset, 121 OTUs were classified as facultative anaerobes or aerobes, 73 OTUs as obligate anaerobes, while 18 OTUs assigned to taxa above genera were categorized as 'unclassified'. The lung microbiome for each CF individual varied from 5 to 44 different OTUs, with the highest richness of obligate anaerobes observed at 17 OTUs per participant (Table 1). Obligate anaerobes were present in 65/70 CF individuals, ranging from 0.1–70.6% of the total microbiome (Fig. 1). A list of OTUs and classification are presented in Tables S1 and S2. The ten most prevalent obligate anaerobes are summarized in Table S3.

Impact of the ten most abundant anaerobic species present in sputum on annual rate of FEV_1 change over an 8 year follow-up period

Two of the ten anaerobic species studied were associated with lung function decline: *Porphyromonas pasteri* and *Prevotella nanceiensis* (Table 2). The effect size of annual rate of FEV_1 decline if these viable organisms were present compared to absent was −52.3 ml yr^{-1} (95% CI:−87.7 to −16.9, P=0.004) and −67.9 ml yr^{-1} (95% CI: −115.6 to −20.1, P=0.005 (Table 2), respectively. Similarly, the relative abundance of live *P. pasteri* and *P. nanceiensis* was associated with a greater annual rate of FEV_1 decline of −3.7 ml yr^{-1} (95% CI: −6.1 to −1.3, P=0.003) and −5.3 ml yr^{-1} (95% CI: −8.7 to −1.9, P=0.002; Table 2) for each $\log_2$ increment of abundance, respectively.

DISCUSSION

This is the first study to prospectively investigate viable anaerobic bacteria present in the sputum microbiota and relationship to long-term outcomes in adults with CF. The presence and relative abundance of both *Porphyromonas pasteri* and *Prevotella nanceiensis* in a cohort predominately colonized with *Pseudomonas aeruginosa* were associated with a greater annual lung function decline over the 8 year study period.

The lower airways in CF are polymicrobial and often contain anaerobes [3–5]. These anaerobes may be the result of repeated micro aspirations of oral flora in combination with an abnormal mucociliary clearance mechanism in CF airways [18], although not all data support this hypothesis [19–21]. For example, Rogers *et al.* demonstrated dissimilarity with paired expectorated sputum and mouthwash samples from the same CF individuals [22]. Despite the high abundance of anaerobes in the CF lower airways, their pathogenicity in the CF airways is not well understood. There is much deliberation over whether obligate anaerobic bacteria, such as *Prevotella* sp., which are known to be pathogenic and to harbour antimicrobial resistance genes [23], can also interact with other bacterial pathogens, such as *P. aeruginosa*, to enhance their virulence and growth [24, 25]. Indeed, evidence suggests *P. aeruginosa*, which preferably uses aerobic respiration, undergoes anaerobiosis in the presence of nitrate in the CF airways [26]. *Prevotella* species can protect *P. aeruginosa* against the activity of ceftazidime, an anti-pseudomonal antibiotic commonly used to treat pulmonary exacerbations [27]. Our data support these observations, suggesting that *Prevotella* sp. may be associated with adverse clinical outcomes. However, our observation that *Porphyromonas* was associated with adverse clinical outcomes is not supported by other studies. Studies in children with CF found that *Porphyromonas* sp. may protect against *P. aeruginosa* colonization [28, 29] and *Porphyromonas* sp. is also less abundant during pulmonary exacerbations, thus suggesting a protective role [28]. However, these studies were both performed in children without *P. aeruginosa*, while our study was performed in adults with chronic pulmonary *P. aeruginosa* and more severe pulmonary disease. We suggest that changes in community dynamics over time, spatial heterogeneity of the lungs and differing microenvironments may account for the contrasting observations. Zemanick *et al.* found that the presence of sputum anaerobes during a pulmonary exacerbation were associated with improved lung function and less inflammation [30], although there was significant variability in anaerobes in response to antibiotics (15). This suggests that diverse anaerobic species present in the airway may have distinct pathogenic or protective roles depending on individual interactions in the lung microbiota [11, 31].

Strengths of the study include the multicentre study population who were recruited from two adult specialist CF centres. Longitudinal follow up over an 8 year period provided robust linear modelling of lung function to predict FEV_1 outcomes. In addition, by using a novel PMA pre-treatment, we were able to give a more accurate estimate of viable anaerobic bacteria.

There are a number of limitations in this study that should be considered when interpreting these data. The enumeration of bacterial cells using the viability PCR technique has not been validated in comparison to standard culture methods for CF sputum [15]. Sputum samples were taken once at baseline only and compared with adverse clinical outcomes therefore findings should be considered as hypothesis generating. Whilst this offers novel insights, longitudinal profiling of the sputum microbiota (including aerobic and facultative anaerobic bacterial species) in a larger cohort and over multiple time points would aid our understanding further on the microbiological anaerobic community dynamics.

Our findings support a potential association of specific anaerobic species abundantly present in CF sputum with long-term lung function decline. These findings suggest that *Prevotella* and *Porphyromonas* species may contribute to lung disease and CF pathophysiology, which should be confirmed with a large, prospective cohort with regular microbiome profiling combined with enhanced validated sequencing strategies.

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Author contributions
K.W. and M.Z. are joint co-authors for this study. The study was designed by K.W., M.Z., H.B., A.F. and I.S. K.W. collected the clinical data, M.Z. and A.L. conducted the microbiome analysis. The statistical analysis was performed by K.W. and I.S. All authors contributed to data interpretation, data presentation and drafting of the manuscript. All authors approved the final version of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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