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Hydrogen cyanide as an *in-vitro* and *in-vivo* marker of *Pseudomonas aeruginosa* infection

Dr Francis J Gilchrist

Submitted for the degree of Doctor of Philosophy

December 2014

Keele University

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ABSTRACT

The work presented in this thesis uses Selected Ion Flow Tube Mass Spectrometry to investigate if hydrogen cyanide (HCN) is a marker of *Pseudomonas aeruginosa* (PA). The initial *in-vitro* studies measure the HCN released into the gas phase by cultures of PA after various durations of incubation.

Study 1 uses clinical PA isolates with a known genotype and phenotype (mucoid / non-mucoid) and Study 2 uses a selection of the same PA isolates cultured under biofilm and planktonic conditions. Study 4 investigates if HCN is an *in-vivo* marker of PA infection in children with cystic fibrosis (CF). It is a 2 year observational study of 233 children with CF who are free from PA infection. A breath sample for HCN analysis is collected each time they attend the out-patient clinic. Exhaled breath HCN concentrations are then compared to routine microbiology sample results. The breath samples for this study are collected in sampling bags. In preparation for this, Study 3 identifies the most appropriate bag type as well as the maximum duration of storage and the need for sample warming prior to analysis.

Some healthy adults produce HCN in their oral cavity and therefore mouth-exhaled HCN alone cannot be used as marker of PA infection. Study 5 investigates nose-exhaled HCN as a marker of chronic PA infection in adults with CF. A recent study has shown that *Burkholderia Cepacia Complex* (BCC) produces cyanide when cultured under biofilm but not planktonic conditions. Study 6 measures the HCN released into the gas phase by *in-vitro* cultures of BCC as well as HCN concentration in the breath of patients with chronic BCC infection.

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TABLE OF CONTENTS

List of Figures

List of Tables

Chapter One

Literature Review

CHAPTER ONE – LITERATURE REVIEW

1.1 CYSTIC FIBROSIS

1.1.1 Background

1.1.1.1 Introduction

Cystic fibrosis (CF) is the commonest life shortening, inherited disease in the Caucasian population. It was first described in 1938[1] and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. These mutations result in defective intracellular processing of the CFTR protein. This is a c-AMP regulated anion channel normally expressed on the apical surfaces of epithelial cells, including those of the sweat glands, pancreas, gastrointestinal tract and reproductive organs.[2] The critical function of CFTR is the transport of chloride ions across membranes but it has a number of other regulatory roles related to bicarbonate and sodium transport, ATP channels, intracellular vesicle transport and acidification of intracellular organelles.[3–7]

The inheritance of CFTR mutations results in a qualitative or quantitative reduction in CFTR activity at the cell surface. The degree of reduction in CFTR activity influences the severity and diversity of the CF disease phenotype.[6] Individuals with a single CFTR mutation (carriers) may have as little as 50% of wild-type CFTR activity but are unaffected. Individuals with CF (two CFTR mutations) will have less severe disease phenotypes if one of their mutations retains residual (but reduced) CFTR function.

Some of the pathophysiological manifestations of CF begin in-utero. These include the effect on the pancreas in which the blockage of ducts leads to auto-

digestion and leakage of enzymes into the systemic circulation.[2] This is the basis of newborn screening programmes which measure the levels of a pancreatic enzyme called immunoreactive trypsinogen (IRT). Reduced CFTR function in the sweat glands results in elevated sweat chloride that is detectable from birth using pilocarpine-induced iontophoresis. Reduced water and bicarbonate secretion in the gut also predisposes neonates to meconium ileus and the majority of males with CF are born without a palpable vas deferens. Other pathologies evolve after birth. Pancreatic insufficiency and the associated gastrointestinal complications are usually present from birth but may not be clinically apparent in the first few years of life. The risk of CF-related diabetes increases with age. Interestingly the respiratory system appears essentially normal at birth but the complications from reduced CFTR function become apparent very quickly.[8,9]

1.1.1.2 The pathophysiology of CF lung disease - lessons from the CF pig

There are a number of hypotheses related to the pathophysiology of CF lung disease but the most widely quoted is the low-volume hypothesis. This suggests that the effect of CFTR dysfunction on sodium channels leads to excess sodium and water resorption which dehydrates the airway surface. The epithelium cannot correct this due to loss of chloride efflux. Decreased periciliary water volume reduces the lubricating layer between epithelium and mucus, which leads to compression of the cilia and impaired mucus clearance.[10] However, studies on CF pigs have challenged this hypothesis by demonstrating a lack of CFTR-dependent changes in sodium absorptive flux, fluid absorption, and depth of periciliary fluid in cystic fibrosis pig airway epithelia.[11] This has led to the suggestion that defects in

chloride and bicarbonate transport, but not sodium transport, are largely responsible for lung disease in cystic fibrosis.

The pathophysiology of CF lung disease was further informed by studies involving CF pigs which showed a reduced ability to kill bacteria that come into contact with the airway surface when compared to wild-type pigs.[12] This was shown to be related to the pH of the airway surface liquid (ASL) which is more acidic in CF pigs. When the ASL pH was reduced in wild-type pigs bacterial killing was reduced and conversely, increasing ASL pH in CF pigs improved bacterial killing.[13] This led to the hypothesis that reduced CFTR function directly effects bicarbonate excretion resulting in a fall in the ASL pH and inhibition of antimicrobial function which impairs the killing of bacteria.

CF pigs have also helped to answer the long-standing question: which comes first, infection or inflammation? Studies have shown that despite the fact that CF pigs develop spontaneous lung infections, neutrophil counts and interleukin (IL)-8 levels in neonatal bronchial alveolar lavage (BAL) fluid do not show signs of inflammation at birth.[14] This suggests that infection precedes inflammation in cystic fibrosis.

1.1.1.3 Genetics

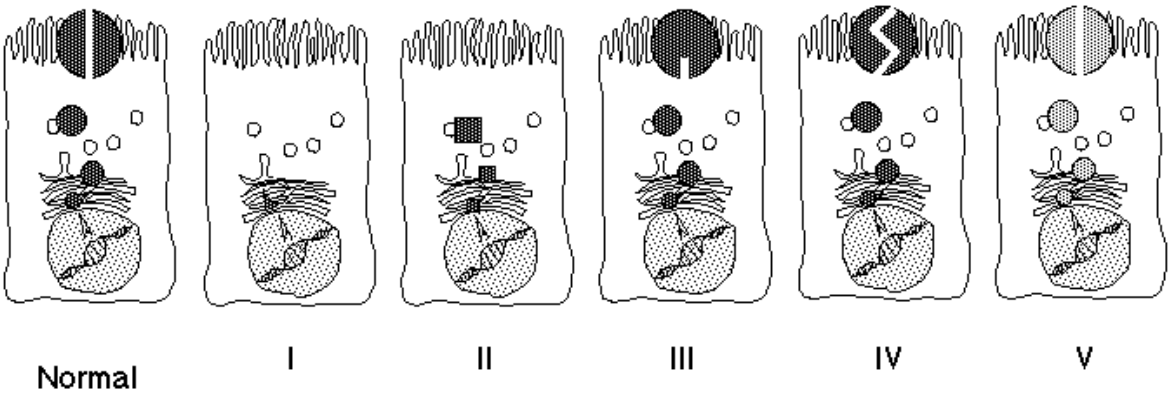
The genetic defect was identified and localised to the long arm of chromosome 7 in 1989[15] and since then, more than 1800 CFTR mutations have been identified.[16] The vast majority of these are confined to one family (private) or a small number of individuals.[17] Worldwide the most common mutation is Phe508del (formally known as ΔF508) which causes a deletion of phenylalanine at the 508th amino acid. Phe508del accounts for nearly 70% of the mutations in the white UK population

meaning that 50% of this population are homozygous for Phe508del. The CF genotype correlates strongly with pancreatic sufficiency and sweat chloride concentrations but poorly for lung function.[18] This means that two patients with the same genotype (including siblings) may have very different manifestations of CF. Factors that influence this phenotypic variability include environmental factors, social factors, chance effects and polymorphisms in non-CFTR genes such as high-producer ACE, transforming growth factor ß1 and mannose-binding lectin-2.[19,20]

1.1.1.4 Mutation classes

CF mutations have been divided into 6 sequential classes according to their functional effects on the CFTR protein.[21,22] See Figure 1. Class I includes mostly nonsense, frameshift or missense mutations resulting in defective protein biosynthesis and non-functional products that are degraded within the cell. Class II mutations, such as Phe508del, produce a misfolded functional CFTR protein, which is prevented from trafficking to the apical surface of the cell. Class III mutations affect channel activation by preventing binding and hydrolysis of ATP. Class IV mutations produce a protein with impaired function because of abnormal anion conduction. Class V mutations result in a reduced number of normally functioning CFTR molecules on the apical surface.[22] Class VI mutations result from truncation of the C-terminus of CFTR and produce a functional protein, which is unstable at the apical membrane surface. As individuals with classes I–III and VI mutations have little or no functional CFTR they are predicted to have severe disease. In contrast, individuals with class IV and V mutations retain some residual CFTR-mediated channel function and would be expected to have milder disease.

Figure 1: Molecular consequences of cystic fibrosis transmembrane conductance regulator mutations.



Used with permission from Wilschanski.[22]

1.1.1.5 Incidence of cystic fibrosis

In the UK, CF has a carrier rate of 1 in 25 and an incidence of 1 in 2500 live births.[23] There is a similar incidence amongst white North Americans (1 in 3300) but it is much less frequent in African-Americans (1 in 15,000), Asian Americans (1 in 31,000) and Japanese (1 in 350,000).[24] In the UK the estimated number of patients with CF is approaching 10,000; it is expected that this number will continue to increase as the number of new cases (300/year) outnumbers the number of deaths (160/year).[25] The outlook for patients with CF continues to improve with the median predicted age of survival increasing from 32 years in 2000 to 37.4 years in 2008 (US CF Foundation Patient Registry data).[26] A recently published UK model predicted a child born today with CF will typically live to be 50 years of age or more.[25]

1.1.2 Diagnosis

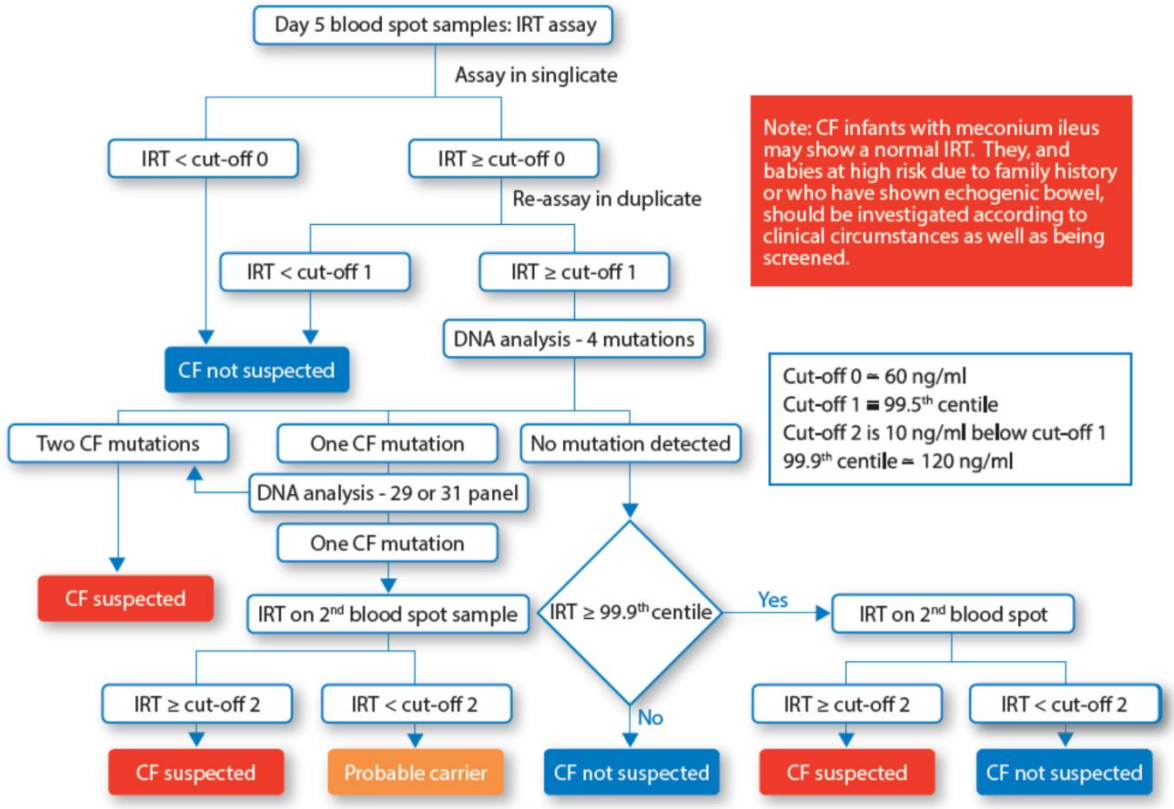
1.1.2.1 Newborn screening

Newborn screening for CF was implemented across the UK in 2007, although it had been running in some areas since 1980. It involves the measurement of immunoreactive trypsinogen (IRT) on the Newborn Bloodspot Screening which is taken on day 5-8 of life. Very high IRT suggests pancreatic injury consistent with but not specific to CF. Various protocols have been used but the current UK protocol is an IRT-DNA protocol.[27] See Figure 2. If the first IRT >99.5th centile, a 4 mutation DNA analysis is undertaken on the same blood spot. If two mutations are identified then the patient is referred to a Paediatric Respiratory Consultant as "CF suspected". If one mutation is identified then a 29-31 mutation DNA analysis is undertaken. If this still only identifies one mutation then a second IRT is measured on a second bloodspot taken on day 21-28 of life. If the IRT >99.5th the patient is referred as "CF suspected", if the IRT is below this level, the patient is a presumed carrier and no further action is taken. If no mutation is detected on the initial 4 mutation DNA analysis, CF is not suspected if the first IRT is <99.9th centile. If the IRT is above this level then a second blood spot is taken and same cut-offs are applied as for the second IRT in the one mutation group.

Although there was a significant financial cost to implementing the newborn screening programme, there is evidence that the treatment costs are lower for patients diagnosed with CF through newborn screening when compared to clinically diagnosed patients.[28] The majority of evidence regarding the clinical benefits of newborn screening for CF is based on the randomised controlled trial in the Wisconsin CF Neonatal Screening Project.[29] This remains controversial as the

patients in the control group did not have their blood spots examined for 4 years, effectively withholding their diagnosis. Despite this, the study has clearly demonstrated improved nutrition in the screened population that is maintained up to the age of 16 years. There was also an increased incidence of vitamin E deficiency amongst the non-screen group and those that were deficient had a significantly lower Cognitive Skills Index. The study has failed to show any significant difference between the groups in terms of lung function but this is confounded by an imbalance of genotype, pancreatic status and severity of lung disease between the two groups and the early acquisition of PA amongst screened patients at one of the centres.[30]

Figure 2: UK standard protocol for neonatal screening for cystic fibrosis



A retrospective, observational study undertaken in Australia has also provided interesting data on the benefits of newborn screening.[31] It compared a cohort of CF patients born in the 3 years prior to the introduction of newborn screening (n=57) with those diagnosed in the first 3 years of screening (n=60). When interpreting the results, it must be noted that the study was not randomised and there was a time gap between the cohorts, At the age of 18 years non-screened patients had a higher rate and lower age of PA acquisition ($p<0.01$). Screened patients had better height, weight, BMI and FEV1 (difference: 16.7 ± 6.4%; $p=0.01$). At age 25 years there was a significant survival difference in favour of the screened group (25 vs 13 deaths or lung transplants; $p=0.01$).

In the UK, the majority of newly diagnosed patients with CF are now identified by the newborn screening programme and the number of late diagnoses has been reduced. Despite this, there can be false negative results with any screening programme and children can therefore still present with CF. This may be because they were born prior to the introduction of the newborn screening programme or they may not be picked-up by the newborn screening programme.[32] The risk of false negative results is higher in non-Europeans, especially Asians as the mutations they carry may not be on the panels used in the UK screening programme. If such patients are from sub-populations with a high incidence of consanguinity then they may be homozygous for rare “private” mutations.[33] There is a higher rate of false negative results in those neonates that present with meconium ileus.[34] The reason for this is not fully understood but it has limited clinical significance as the possibility of CF is highlighted by the presence of meconium ileus.

1.1.2.2 Sweat test

Despite the introduction of newborn screening, the sweat test remains the gold standard diagnostic test for CF. There are strict guidelines regarding the methodology which is based on pilocarpine iontophoresis and a quantitative determination of chloride concentration.[35] A sweat chloride concentration of >60mmol/L is diagnostic of CF. In infants with an intermediate result (sweat chloride: 30-60mmol/L) the diagnosis may be confirmed or refuted based on additional data from further mutation analysis, a repeat sweat test or clinical assessment. In some infants the diagnosis remains equivocal. European consensus on the follow-up and management of these children is currently being agreed[36]. False negative sweat test results are reported in the literature but are more frequently related to poor technique. Textbooks provide a long list of potential causes of false positive sweat tests, but in reality the only causes that are likely to be encountered are severe skin disorders (eczema), malnutrition and certain immunodeficiency states.

1.1.3 Clinical Manifestations

1.1.3.1 Respiratory

Cystic fibrosis is characterised by recurrent lower respiratory infections and chronic inflammation leading to progressive lung damage and respiratory failure. Studies analysing BAL samples in infants diagnosed by newborn screening have demonstrated that this process starts in the first few months of life.[8,37] These studies found evidence of infection (positive cultures for *Staphylococcus aureus* and PA) and inflammation (elevated neutrophil count and proinflammatory

cytokines) in infants as young as 2 months. This is despite the majority of these infants being asymptomatic.

In infants the commonest organisms to infect and colonise the lower respiratory tract are *Staphylococcus aureus* and *Haemophilus influenzae*. As the patients get older other organisms become established; of significant importance are PA (this will be discussed in more detail later in this chapter) and *Burkholderia Cepacia Complex* (BCC), which have well documented effects on morbidity and mortality. Chronic infection with a range of other organisms whose pathogenicity is less well understood (including methicillin resistant *Staphylococcus aureus* (MRSA) and atypical mycobacterium) is also common. It is often difficult to establish if isolation of these organisms is related to colonisation or invasive infection. The incidence of MRSA is much higher in the United States (21%) than in the UK (8%).[26,38] Interestingly, prophylactic antibiotics against *Staphylococcus aureus* are not used routinely in paediatric patients in the United States. Persistent infection with organisms such as PA leads to the production of chemotactic cytokines which recruit polymorphonuclear cells. The recruited neutrophils are damaged by toxins and elastases released by the infecting bacteria, which leads to the attraction of further inflammatory cells and lung cell damage.[4] The release of DNA from the damaged neutrophils adds to sputum viscosity.

Fungi and yeasts are frequently isolated from the sputum of patients with CF, the significance of which is not always understood. *Aspergillus fumigatus* rarely causes invasive disease or aspergillomas in CF but does commonly case allergic bronchopulmonary aspergillosis (ABPA). ABPA is a hypersensitivity reaction to *Aspergillus fumigatus* leading to a Th2 CD4 response mediated by the release of

specific IgE.[39] It can cause severe impairment to lung function and permanent lung damage; early diagnosis and treatment is therefore vital. Symptoms include wheeze and respiratory deterioration not responsive to antibiotics. Diagnosis is based on total IgE, specific IgE to *Aspergillus fumigatus* or skin prick tests, *Aspergillus* precipitins and radiology. Treatment is oral corticosteroids and antifungals.

1.1.3.2 Gastroenterology

Prior to CF newborn screening the commonest reason for early diagnosis was meconium ileus, which causes intestinal obstruction secondary to inspissated meconium, affecting about 15% of infants with CF. The vast majority (85-90%) of patients with CF have exocrine pancreatic insufficiency which causes steatorrhoea, malabsorption, malnutrition and deficiency of fat soluble vitamins (A, D, E & K). Clinically evident cirrhosis develops in about 5% of CF patients secondary to focal biliary cirrhosis caused by obstruction of intrahepatic bile ducts.[4]

1.1.3.3 Endocrinology

Cystic fibrosis related diabetes (CFRD) develops when enough of the pancreatic islet cells are not functioning to cause insulin insufficiency and carbohydrate intolerance, with insulin resistance also potentially contributing. The pancreatic dysfunction is caused by thick secretions obstructing the intra-pancreatic ducts and eventually autolysis.[4,40] CFRD is associated with respiratory deterioration, more severe exacerbations and poor nutritional status. Clinically apparent CFRD is present in 30% of CF patients aged over 25 years[26] but abnormal glucose metabolism has been identified in 38% of asymptomatic adolescents.[41] Given this

information it is necessary to screen cystic fibrosis patients for CFRD on a regular basis; although most centres use the oral glucose tolerance test there is debate regarding what is the best screening tool and the age at which screening should start.[42]

1.1.3.4 Bones

Bone disease and its associated complications are increasingly being recognised in CF. The prevalence of osteopenia and osteoporosis bone disease in adults with CF is 38% and 24% respectively.[43] Regarding complications, the prevalence of vertebral fractures and non-vertebral fractures is 14% in those with osteopenia and 20% for those with osteoporosis.[43] Bone disease in CF is caused by a combination of factors including: vitamin D deficiency, corticosteroid use and chronic systemic inflammation. Oral and intravenous bisphosphonates have been shown to increase bone mineral density in patients with CF and are increasingly being used.[44]

1.1.3.5 Reproduction

The vas deferens is very sensitive to CFTR dysfunction. Virtually all men with CF and a percentage of male CF carriers have congenital, bilateral absence of the vas deferens.[45] This results in azoospemia and infertility. Women with CF often have reduced fertility secondary to poor nutritional status and increased viscosity of cervical mucus.[46] Despite this they can conceive naturally and if nutrition, glycaemic control and lung function is maximised a successful pregnancy can be completed.[47]

1.1.4 Treatment

1.1.4.1 Nutrition

It has long been established that optimising nutrition in patients with CF improves lung function and survival.[48] In those who have exocrine pancreatic insufficiency this is done by the replacement of pancreatic enzymes and fat soluble vitamins. Regular input is required from a dietician to closely monitor growth and ensure a high calorie diet. If the growth or nutritional status is suboptimal then additional nutritional support, including overnight enteral feeds may be required.

1.1.4.2 Chronic pulmonary management

In the UK prophylactic oral antibiotics against *Staphylococcus aureus* are given for at least the first 2 years. Oral macrolide antibiotics, especially azithromycin are widely used as a prophylaxis in older children and adults. Azithromycin has been shown to reduce pulmonary exacerbations and improve FEV_1 in patients with chronic PA,[49] in patients who are PA negative it reduces exacerbations but does not improve lung function.[50] The exact mechanism is unclear but azithromycin is known to have bactericidal effects and decrease production of biofilms and virulence factors by PA. It also has an anti-inflammatory role by affecting cytokine production and polymorphonuclear cell function.[51] Long term, low dose azithromycin has been associated with hearing loss.[52,53] There are concerns that chronic macrolide use may predispose to infection with atypical mycobacterium or increase the likelihood of such an infection developing antibiotic resistance.[54,55]

In patients with chronic PA there is evidence that nebulised anti-*Pseudomonas* antibiotics (colistin or tobramycin) improve lung function, decrease PA density in

sputum and reduced hospital admissions.[56,57] As yet, there is little long-term data to determine if these benefits are maintained long term.[58] Nebulised mucolytics have been shown to improve lung function and decrease pulmonary exacerbations.[59,60] Dornase alpha is an enzyme that cleaves DNA, by hydrolysing the neutrophil DNA present in CF sputum it reduces the viscosity allowing easier mucociliary clearance.[60] Hypertonic saline draws water into the airways rehydrating the periciliary layer, which can also improve mucociliary clearance.[59]

1.1.4.3 Pulmonary exacerbations

Early and aggressive treatment of infective pulmonary exacerbations using oral or intravenous (IV) antibiotics has been shown to improve lung function and survival.[61,62] The choice of antibiotic will depend on the organisms cultured by individual patients, their previous response to treatment and the presence of hypersensitivity reactions to certain antibiotics. When using intravenous antibiotics against PA, a combination of agents with different modes of action is preferred to single agent treatment to avoid the emergence of resistant strains.[63] Although there is a paucity of data regarding the optimal duration of treatment, most patients receive around 14 days.[64] When specific organisms are isolated for the first time, such as PA, BCC or methicillin resistant *Staphylococcus aureus,* eradication regimens using a combination of intravenous, oral or inhaled antibiotics should be used. If successful this will avoid the morbidity and mortality associated with chronic infection.[38,65–67]

1.1.4.4 Airway clearance

Airway clearance techniques are chest physiotherapy treatments which help to loosen sputum to enable it to be cleared by coughing or huffing. In young children

the main technique is percussion and drainage that is performed by the parent. For older children and adults devices and techniques are used to allow independent treatment. There is evidence of the benefit from airway clearance techniques in CF but no single method has been shown to be more advantageous than the others.[68] Exercise has been shown to be beneficial for fitness and general well-being but there is no evidence it should be used as an alternative to airway clearance.[69]

1.1.4.5 Lung transplantation

Lung transplantation is the final therapeutic option for patients with end stage disease. There has recently been much controversy about this topic after a paper from the United States suggested only 5 of the 514 children with CF listed for transplantation would have received survival benefit from the procedure.[70] Subsequent articles have strongly refuted this analysis and suggested that if patients are appropriately selected for transplantation it has the potential to increase survival benefit.[71,72] In Europe it is unusual for children to be listed for transplant unless their projected life expectancy is less than two years despite maximal medical therapy.[71] Adults tend to be considered for transplant when their FEV_1 plateau below 30% predicted. Irrespective of the criteria for listing patients for transplantation, the procedure does not offer a cure and the 5 year survival post-transplant for children is less than 50% with only slightly better outcomes in adults.[70–72]

1.1.4.6 Gene therapy

Since the CFTR gene was cloned in 1989 there has been huge interest in the possibility of gene therapy providing a cure for CF.[15] Gene therapy is the process

by which a normal copy of the gene is introduced into the target organ by a gene transfer agent, which can be a viral or a non-viral vector. Theoretically CF is an ideal target for gene therapy as it is the result of a single gene defect and the airway epithelium can be targeted with aerosols.[73] The first report of patients receiving CFTR gene therapy appeared in 1993 and there have been more than 20 subsequent clinical trials using various viral and non-viral vectors. These were predominantly single doses administered into the nose or lungs.[74] Some of these studies demonstrated an improvement of chloride channel function but this was generally short-lived. The UK CF Gene Therapy Consortium was founded in 2001 to coordinate gene therapy in the UK. They have developed a translation programme with two products (Wave 1 based around liposomal gene transfer and Wave 2 focused on a modified lentivirus). The Wave 1 product has been used in the UK CF Gene Therapy Multidose trial and more than half of the recruited patients have now received their 12 treatment / placebo doses. Development of the Wave 2 product is continuing and the first clinical trials are planned for 2017.

1.1.4.7 Mutation specific therapies

There has been significant research of therapies specific to certain mutations. These include ‘correctors’, which are agents that correct the localisation of CFTR by increasing its density at the cell membrane and ‘potentiators’ which are agents that increase the function of CFTR correctly located at the cell membrane.[4]

Ivacaftor is a potentiator used in patients with at least one G551D CFTR mutation. An initial study to assess the safety and side-effect profile of ivacaftor showed that after 28 days treatment there were significant improvements in lung function (FEV1) and CFTR function (nasal potential difference and sweat chloride).[75]

There were no concerns regarding its safety or side-effect profile. Subsequently a 48 week, randomised, double blind, placebo controlled trial of ivacaftor was undertaken in patients >12 years. This showed highly significant improvements in CFTR function (sweat chloride decreased by 45 mmol/L at 3 weeks and maintained at 48 weeks), FEV_1 (increased by 10.6% at 24 weeks and maintained at 48 weeks), weight (increased by 3.1kg at 48 weeks), pulmonary exacerbation (26% fewer exacerbations by 48 weeks) and quality of life.[76]

In another randomised, double-blind, placebo controlled trial in children aged 6-12 years, similar improvements were seen despite them being healthier with near normal baseline lung function and nutrition.[77] Ivacaftor was approved by the European Medicines Agency in July 2012 and in February 2013 it was announced that the NHS would fund the treatment for all patients in England aged 6 years or over with at least one G551D gene mutation. It is estimated that there are 270 eligible patients in England.

In patients homozygous for the Phe508del mutation, ivacaftor produce no significant improvement in FEV_1 and the mean sweat chloride at week 16 only reduced by 2.9 mmol/L.[78] The potentiator VX-809 also produced a dose-dependent reduction in sweat chloride but no significant improvements in FEV_1.[79] The results of a phase 3 trial using a combination of ivacaftor and VX-809 in patients homozygous for Phe508del are awaited. There is much hope that a mutation-specific therapy for patients with the Phe508del mutation can be developed to produce similar outcomes as seen in those with a G551D mutation who receive ivacaftor. A large volume of research is being undertaken in this area but so far the results have been frustrating.

1.2 *PSEUDOMONAS AERUGINOSA* IN CYSTIC FIBROSIS

1.2.1 Bacteriology

Pseudomonas aeruginosa (PA) is a gram-negative rod shaped bacterium which belongs to the γ proteobacteria class of bacteria.[80] Its ability to thrive in normal or hypoxic conditions means it can occupy a wide range of niches; from an almost ubiquitous presence in the environment to causing infection in a variety of animals and plants. In the environment it is present in soil and water and can colonise surfaces, including those of medical equipment.[81] Its ability to cause infection across species and across kingdoms is unusual and demonstrates its versatility. PA is the epitome of an opportunistic pathogen of humans. It almost never infects uncompromised tissues, but if there is a break in the host's defences PA will exploit it and can cause infection in almost any tissue. These include infections in patients with AIDS, neutropenia and those mechanically ventilated.[82,83] PA is a leading cause of hospital acquired infection, accounting for approximately 10%.[84]

1.2.2 Acquisition of *Pseudomonas aeruginosa* in Patients with Cystic Fibrosis

Although most patients with CF are infected by their own individual PA strain, siblings often carry the same strain indicating infection from a common environmental strain or from cross infection.[85] Recent microbiological surveillance using molecular typing (genotyping), has provided compelling evidence for PA cross-infection at many European, Australian and Canadian CF centres.[86–89] These strains are often resistant to multiple antibiotics and therefore difficult to eradicate.[90] The emergence of transmissible PA strains has had a major impact

on the infection control precautions necessary for patients with CF and further highlights the need for prompt, accurate PA diagnosis.

1.2.3 Survival Mechanisms

PA uses a number of innovative survival mechanisms to enhance survival in the CF airway and establish chronicity.

1.2.3.1 Evading the host immune system

PA secretes a number of products to aid survival. These included pyocyanin which slows ciliary beat frequency thereby impairing mucociliary clearance and exotoxin A which inhibits phagocytosis. Elastase and alkaline protease are also secreted which cleave immunoglobulin's, cytokines and complement.[91,92] The release of pro-inflammatory markers such as IL-8 and stimulates the host immune response via Toll-like receptor 5.[93]

1.2.3.2 Development of a mucoid phenotype

Initial PA infection usually occurs with a non-mucoid strain that is sensitive to anti-PA antibiotics.[94] As PA is exposed to various antimicrobials over time, it accumulates mutations that help establish chronic infection and antibiotic resistance.[91] These include the mutation in *mucA* which negatively regulates the production of alginate. The increased production of this exopolysaccharide results in a mucoid phenotype when grown *in-vitro*.

Alginate has a number of pathogenic roles:

(i) It acts as a direct barrier against phagocytic cells such as neutrophils and macrophages.

(ii) It has an immunomodulation role and affects the oxidative burst of leukocytes.

(iii) It has a role in the development of biofilms affecting bacterial adhesion and antibiotic resistance.[80,95,96]

The clinical effects of the change to a mucoid phenotype include increased inflammation, a decline in lung function and increased morbidity and mortality.[97] Once the PA phenotype has changed to mucoid, attempts to eradicate the PA infection are much less likely to be successful.[79]

1.2.3.3 Biofilm formation

During early infection, PA attaches to the respiratory epithelium and multiplies whilst in a planktonic (free-floating) form. Once a certain bacterial density is reached, growth slows and biofilm production is signalled. This signalling occurs by quorum sensing in which extra-cellular molecules are used to regulate phenotype in response to population density. Biofilms are communities of non-motile organisms embedded in an exopolysaccharide matrix attached to a solid surface.[98] When growing in this form, PA is more difficult to remove by mucociliary clearance and has increased antibiotic resistance.[99] A mucoid phenotype is a marker of biofilm formation.[100]

The antibiotic resistance is created by restricted antimicrobial penetration mediated by the polysaccharide matrix formation, a reduction in growth and metabolic activity caused by nutrient and oxygen gradients, increased expression of multidrug efflux pumps and an induction of the bacteria general stress response, which increases resistance to environmental stressors.[98] Bacteria in biofilms can be up to 1000 times more resistant to antimicrobials than planktonic

bacteria.[81] Biofilm PA also undergoes physiological, metabolic and phenotypic changes leading to a biofilm-specific phenotype.

1.2.4 Detecting *Pseudomonas aeruginosa* in Patients with Cystic Fibrosis

The culture of PA from expectorated sputum is the gold standard for the detection of PA in patients with CF. Unfortunately, most young children and a significant proportion of older children and adults are unable or unwilling to expectorate sputum. In such patients, clinicians have to rely on other detection methods which are less effective. The most widely used method is the oropharyngeal (cough swab) culture. Although this has a good negative predictive value (95-97%), the sensitivity (44-82%), specificity (83-95%) and positive predictive value (41-44%) are lower.[101,102]

The expectoration of sputum can also be induced in patients who do not usually produce sputum by inhalation of hypertonic saline. Traditionally, paediatricians have thought that this was too unpleasant to be widely used in children but recent studies revealed it to be well tolerated and to produce a good microbiological yield.[103,104] These studies found the factors limiting its routine use were the time required (30-85 minutes) and the expense ($150 per induction). Culture of BAL samples taken during flexible bronchoscopy is increasingly being used to gain a microbiological diagnosis in non-expectorating children.[105] Although this gives an excellent yield its frequency of use is limited because of its invasive nature and the requirement for deep sedation or a general anaesthetic. Despite its increasing use, a study comparing outcomes from standard therapy versus BAL directed therapy in children with CF did not show any difference in the prevalence of PA infection or computerised tomography (CT) scores up to the age of 5 years.[106]

There is also evidence that the bacterial distribution within the lungs of patients with CF is inhomogeneous and the microbiological yield is therefore dependent on which lobes are sampled.[107]

There is interest in the role of serological markers of the immune response to PA antigens including crossed immunoelectrophoresis, radioimmunoassay and enzyme-linked immunosorbent assay. As yet these techniques are not widely used.[63] Anti-PA antibody titres may be helpful in distinguishing early from chronic infection[108] and may be positive before PA is cultured.[109] Although some centres include anti-PA antibody titres as part of a patient's annual review the test is not suitably sensitive or specific to be widely used. There is increasing research using molecular techniques such as polymerase chain reaction (PCR) for PA detection. Initial studies suggest this technique is sensitive (97%) but not specific (46%).[110] There is therefore an urgent need for a non-invasive, child friendly method of detecting early PA infection in children who cannot expectorate sputum.

1.2.5 Classification of *Pseudomonas aeruginosa* Infection

When reading the literature the terminology regarding the various categories of PA can be confusing as different terms are used interchangeably by different authors. The most comprehensive and widely used is the Leeds criteria.[111] This divides patients into 4 categories based on their airway culture results from the previous 12 months:

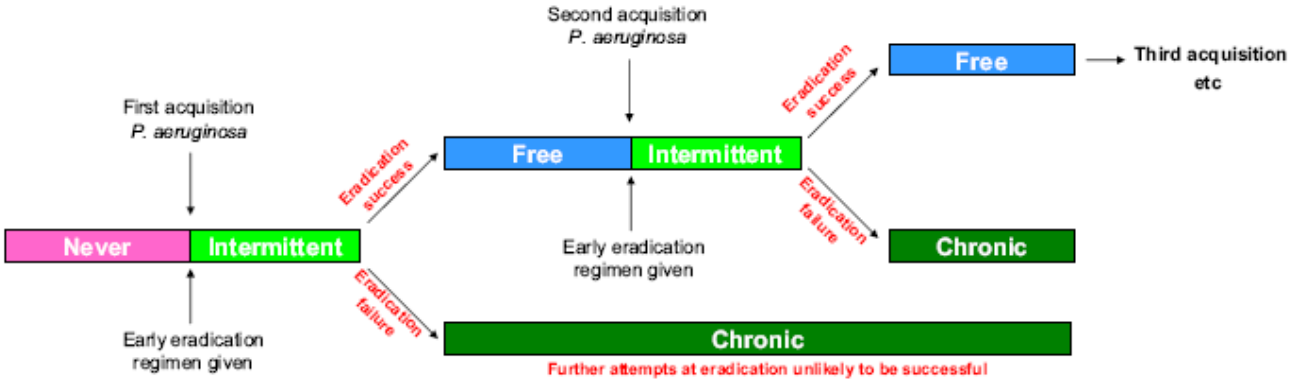
i. Never: PA never cultured
ii. Free of infection: No growth of PA in previous 12 months having had previous PA positive culture

iii. Intermittent infection: <50% of cultures positive for PA in the previous 12 months

iv. Chronic infection: >50% of cultures positive for PA in the previous 12 months

The time course of PA infection in patients with CF is illustrated in Figure 3.

Figure 3: Illustration of the time course of *Pseudomonas aeruginosa* infection in patients with cystic fibrosis using the Leeds criteria.



Used with permission from Lee.[112]

An American longitudinal cohort study showed that over 97% of patients had serological or microbiological evidence of PA infection by age 3.[113] More recently the AREST-CF group published data on 116 children diagnosed with CF by newborn screening between 1999 and 2008. They had flexible bronchoscopy and BAL at diagnosis and annually thereafter.[114] PA was detected in 33/116 (28%) of children and the median (range) age at detection was 30.5 (3.3-71.4) months. At detection, the PA was mucoid in 6 (18%) children and less than 50% of the children were symptomatic at the time of detection.

1.2.6 Clinical Implications of *Pseudomonas aeruginosa* Infection

In patients with CF, chronic PA infection is associated with significant morbidity and mortality. Patient with chronic PA infection have more severe lung disease assessed using chest x-rays when compared to those who are free of PA infection.[115] This results in more symptoms and a more rapid decline in lung function.[116,117] Patients with chronic PA also have more in-patient hospital days and a higher treatment burden due to the additional use of nebulised treatments.[118] A large registry-based study showed it to be the main predictor of morbidity and mortality in young children with CF, the 8-year risk of death was 2.6 times higher in those infected with PA compared to those that were not.[119] In 2007 the life expectancy in the US was 30 years for a patient with CF and chronic PA infection compared to 40 years for a patient with CF free from PA infection.[26] This results in a lower life expectancy It is therefore vital that PA is diagnosed early and if possible eradicated, thereby avoiding chronic infection and its associated morbidity and mortality.

1.2.7 Eradication of *Pseudomonas aeruginosa* Infection

Initial PA infection usually occurs with a non-mucoid strain which is sensitive to anti-pseudomonal antibiotics thereby making eradication possible.[94] Three small randomised controlled trials and a subsequent Cochrane Review[65,120–122] have demonstrated that early eradication regimens against PA do significantly improve the clearance of PA and reduce the prevalence of chronic PA infection 2 years later. The AREST-CF publication discussed earlier had a PA clearance rate of 77% after one course of eradication treatment and 89% when a second course was given to those who had failed the first.[114] The PA eradication regimens have

differed between studies, using various combinations of oral, nebulised and intravenous antibiotics.[123–127] The optimum regimen is unclear and there is currently a large multicentre randomised controlled trial assessing PA eradication with IV and nebulised versus oral and nebulised antibiotics (TORPEDO-CF).

1.3 CYANIDE PRODUCTION BY *PSEUDOMONAS AERUGINOSA*

1.3.1 Background

One of the intriguing aspects of the biology of PA is its ability to produce cyanide. This was first described as long ago as 1913 under its former name *Bacillus pyocyaneus.*[128] PA is one of a limited number of organisms that can synthesise cyanide, the other organisms traditionally known to be cyanogenic are: *Pseudomonas fluorescens, Pseudomonas aureofaciens, Pseudomonas chlororaphis, Chromobacterium violaceum*, and *Rhizobium leguminosarum.*[80,129] These organisms are not usually isolated from patients with CF. More recently, BCC has also been shown to produce cyanide under biofilm but not planktonic conditions.[130] BCC is found in the lungs of patients with CF but it is usually acquired late in the course of the disease and is therefore uncommon in children.

1.3.2 Conditions for *Pseudomonas aeruginosa* Cyanogenesis

Cyanide synthesis occurs by the oxidative decarboxylation of glycine by a hydrogen cyanide synthase enzyme, this process also produces four electrons and four hydrogens per glycine molecule.[80] Cyanide synthesis is maximised under microaerobic (O_2 <5%) conditions and is almost completely abolished under anaerobic conditions.[131] Cyanide production by PA is maximised at temperatures between 34^{o}C and 37^{o}C.[132]

1.3.3 Methods of Cyanide Detection

Using a cyanide ion-selective electrode to measure the non-volatile cyanide (CN^-) ions in aqueous solution, cyanide levels of 300-500μM have been found in PA cultures[129,132] and up to 130μM in the sputum of CF and non-CF bronchiectasis patients with PA infection.[133,134] Conversely, cyanide is essentially absent from the sputum of CF and non-CF bronchiectasis patients without PA infection and from the sputum of healthy controls.[133,134] The volatile compound hydrogen cyanide (HCN) is mainly undissociated when in aqueous solution at a neutral pH and is therefore readily released into the headspace over PA cultures. Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) has therefore been used to measure gas phase HCN. Using this methodology the HCN concentration has exceeded 17,000 parts-per-billion by volume (ppbv) in the headspace (volume 200ml) over PA cultures[135] and 60ppbv in the breath of CF patients infected with PA.[136] HCN is also very low or absent in the breath of healthy children.[136,137]

1.3.4 Explanation for *Pseudomonas aeruginosa* Cyanogenesis

Cyanide is highly toxic, rapidly diffusing through tissues to irreversibly bind to the terminal oxidases of aerobic respiratory chains inhibiting aerobic respiration. PA seems to avoid the toxic effects of cyanide by active detoxification mechanisms and synthesis of a respiratory chain terminated by a terminal oxidase that is insensitive to cyanide.[138–141] The exact reason why PA produces cyanide is unclear but it is likely that it provides an advantage in the ecological niches it inhabits.[80] One such advantage is its role in the exclusion of other lung pathogens, leading to PA becoming the dominant bacterium. This is supported by studies showing cyanide to be the mediating factor in the paralytic killing model of

Caenorhabditis elegans by PA.[142] Cyanide production may also have a role in the pathogenicity of PA as the concentrations of cyanide identified in the sputum and breath are high enough to affect lung cellular function and contribute to the lung damage caused by PA infection.[133,134,136]

1.3.5 *Pseudomonas aeruginosa*, Cyanide and Quorum Sensing

PA is one of many organisms that employ quorum sensing; a process by which extracellular molecules are used to regulate phenotypes in response to population density. In PA, cyanide production is regulated in part by quorum sensing, with high population densities inducing cyanide synthesis.[143–145] The high PA population density of CF mucus maximises quorum sensing cyanide production and together with its low O_2 concentration and ideal temperature, provides the perfect environmental parameters for PA cyanogenesis. Although high PA population densities up-regulate cyanide production by quorum sensing, a study measuring the cyanide levels in the sputum of CF and non-CF patients with bronchiectasis showed cyanide levels to be independent of the PA bacterial load.[133] This, and the absence of cyanide from a small number of sputum / breath samples from PA infected patients, suggest that cyanide production is dependent on the presence of specific PA strains as well as the total PA load.[133,134,136] The theory that cyanide production varies between PA genotypes is supported by the finding that the Liverpool epidemic PA strain overproduces certain quorum sensing regulated exoproducts.[146] No study to date has assessed the variation in cyanide production by different PA genotypes.

1.3.6 Possible Other Sources of Cyanide

It must be considered that cyanide detected in patients breath or sputum is due to cyanogenesis from non-microbial sources. Human leukocytes challenged with *Staphylococcus epidermidis* have been reported to produce hydrogen cyanide *in-vitro* and it is possible that the airway inflammation caused by PA infection results in cyanide production by leukocytes.[147–149] This is supported by a recent study from the AREST CF team who found neutrophil number in BAL samples to be a predictor of cyanide concentration.[150] However, it should be remembered that the BAL neutrophil count is significantly higher in patients infected with PA compared to those with no infection or infection with other organisms.[37] Also, if cyanide was produced by leukocytes, it would be expected to be identified in the sputum or breath of patients infected with organisms other than PA and previous studies have failed to show this.[133,134,136] The high percentage of PA negative patients in whom cyanide was identified in the AREST-CF study is likely to be the reason the authors were unable to use cyanide to differentiate between the patients who had PA infection and those they believed to be free from PA. Future research could help to clarify the role of leukocytes in cyanide production related to PA infection.

1.4 THE ROLE OF SELECTED ION FLOW TUBE MASS SPECTROMETRY (SIFT-MS)

1.4.1 Hydrogen Cyanide as an *In-vitro* Marker of *Pseudomonas aeruginosa*

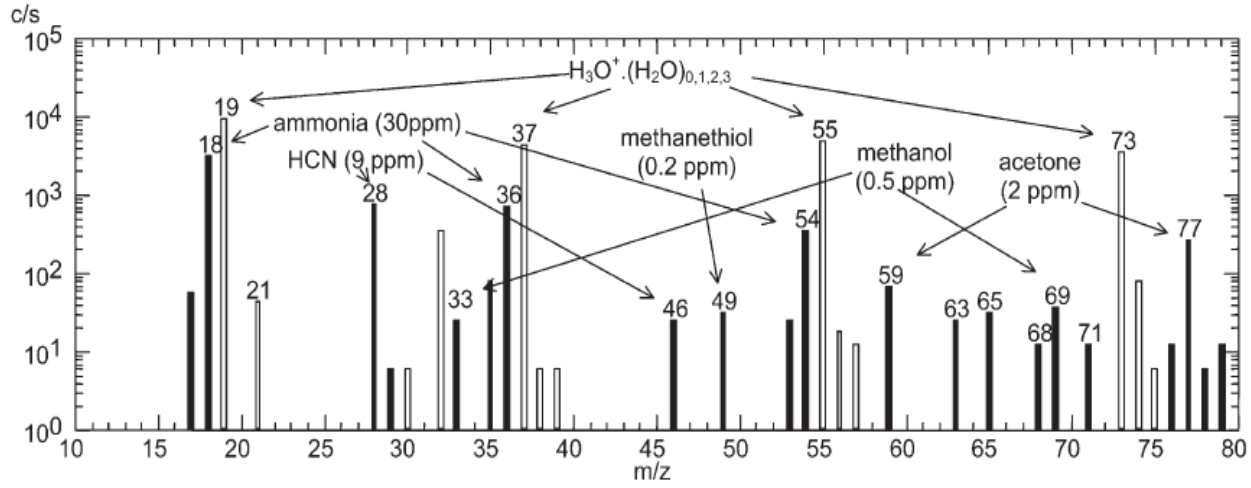
The hypothesis that an exhaled breath marker, specific to PA may be identifiable was generated by Professors Lenney and Smith due to a number of parents commenting that when their child had a PA chest infection they had associated

halitosis. Due to the difficulty in diagnosing PA in non-expectorating children, this possibility was very appealing.

Using selected ion flow tube mass spectrometry (SIFT-MS) which was developed at Keele University,[151,152] *In-vitro* analysis of the headspace of PA cultures was undertaken to identify volatile compounds released into the gas phase. Using the H_3O^+ precursor ions, the SIFT-MS spectrum consistently showed product ions with mass-to-charge ratios (*m/z*) of 28 and 46 in the headspace of the PA cultures and not the control cultures (see Figure 4). The product ion with an *m/z* value of 28 was identified as protonated HCN (H_2CN^-). $C_2H_4^+$, N_2^+ and CO^+ were excluded as possibilities as they cannot be formed in an exothermic reaction of H_2O^+ with a stable chemical compound and hydrogen isocyanide (HNC) was excluded as it was thought highly improbable that PA would produce this less stable isomer. This meant that the product ion with an m/z value of 46 must be $HCNH^+H_2O$.[153] Further analysis of the ion chemistry of HCN allowed the required SIFT-MS kinetics database to be constructed, thereby allowing accurate quantification of gaseous HCN in moist air samples.

The identification of these product ions confirmed the presence of HCN in the headspace of PA cultures. Although, it had been known for a number of years that PA has the ability to produce cyanide, this was the first study to detect HCN released into the gas phase by PA cultures identifying it as a possible marker of PA infection identifiable in exhaled breath.

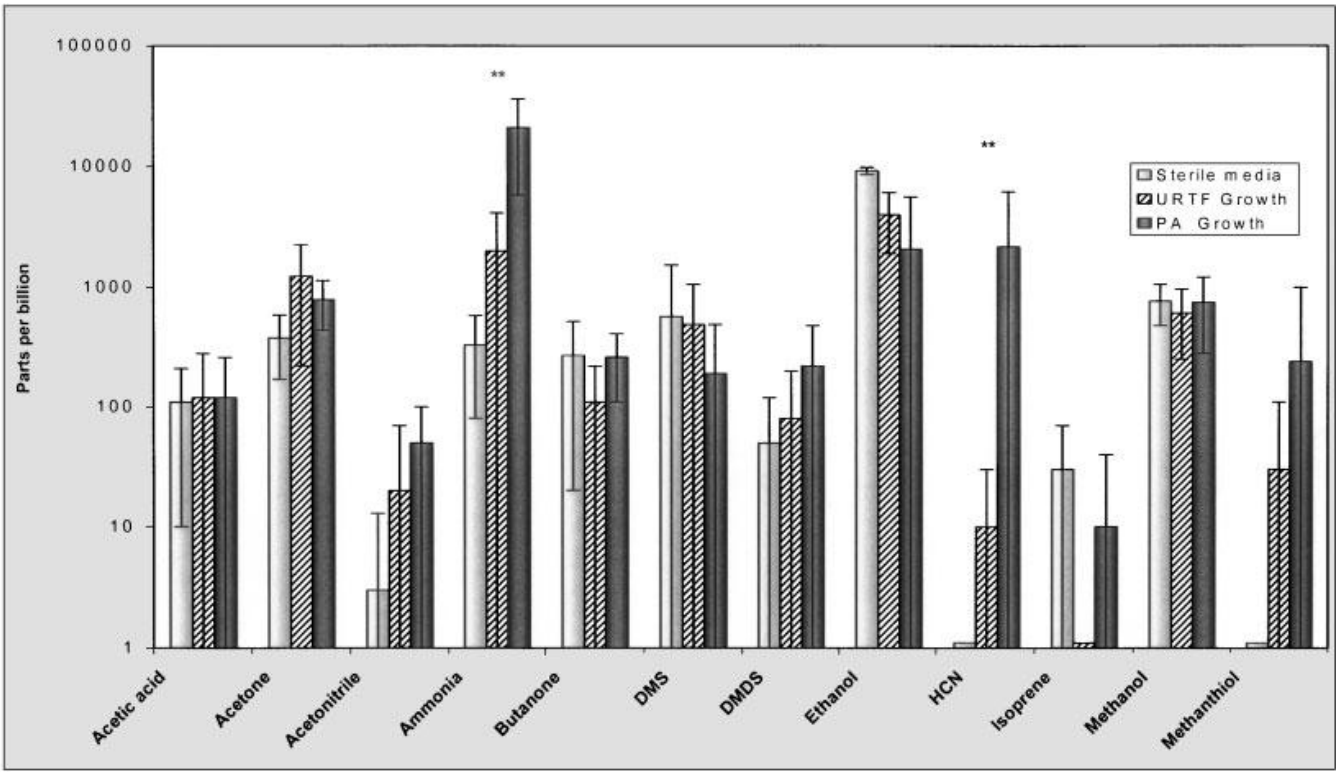
Figure 4: SIFT-MS spectra of *Pseudomonas aeruginosa* culture headspace using H_3O^+ precursor ions.



Expected compounds in culture headspace included ammonia, methanethiol, methanol and acetone. Compounds have multiple peaks due to the addition of water molecules. The peaks at m/z 28 and 46 were unexpected and identified as HCN. Used with permission from Spăněl.[154]

In total, 22 PA cultures and 13 control cultures (6 sterile plates and 7 cultures of mixed upper respiratory tract flora [URTF]) were analysed after 48 hours incubation.[135] HCN was detected in the headspace of 15/22 PA and 1/7 URTF cultures. The mean concentration of the 15 positive PA cultures was significantly higher than the concentration of the single positive URTF culture (2170ppbv v 60ppbv; $p<0.01$). (Figure 5). Using a cut-off of 100ppbv this gave a sensitivity of 68% and a specificity of 100%. Other compounds were identified but were not specific to PA.

Figure 5: Volatile compounds detected in headspace of *Pseudomonas aeruginosa*, upper respiratory tract flora and blank cultures.

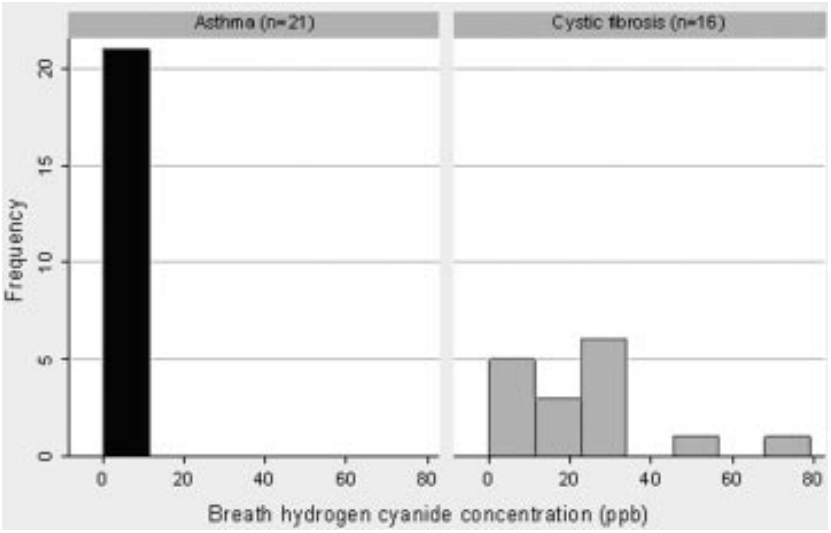


Used with permission from Carrol.[135]

1.4.2 Hydrogen Cyanide as an *in-vivo* marker for *Pseudomonas aeruginosa*

Once SIFT-MS demonstrated that HCN is released into the gas phase by PA cultures *in-vitro* it was hypothesised that HCN would be detectable in the breath of patients with CF and PA infection. To test this hypothesis multiple breath samples were taken from 16 patients with CF and PA infection and 21 patients with asthma.[136] Children with CF had higher median HCN concentrations than those with asthma: 13.5 parts per billion by volume (ppbv) (IQR 8.1–16.5) versus 2.0 ppbv (IQR 0.0–4.8), $p<0.001$. See Figure 6. Intra-subject variability was high and significant changes in HCN concentrations were not observed related to changes in lung function or clinical status.

Figure 6: Exhaled breath hydrogen cyanide concentration at first visit.



Used with permission from Enderby.[136]

1.4.3 Breath Hydrogen Cyanide in the Healthy Population

Using SIFT-MS the HCN concentration in the mouth-exhaled breath of 200 healthy children aged 7-18 years was measured.[137] The vast majority (90%) of healthy children had undetectable (<2ppbv) HCN concentrations and the mean HCN concentration in the 20 children with detectable concentrations was only 8ppbv. In contrast, healthy adults have been shown to have mouth-exhaled HCN concentrations of up to 60 ppbv.[155,156] When mouth and nose-exhaled HCN concentrations are measured simultaneously in healthy adults, the nose-exhaled HCN concentration remains very low or undetectable even when the mouth-exhaled HCN concentration is high. This suggests that in contrast to healthy children, healthy adults may generate some HCN in the oral cavity and if breath HCN is to be used as a biomarker in adults this should be measured in nose-exhaled breath.[156]

1.4.4 Other Applications of SIFT-MS

In addition to its role investigating PA cyanogenesis, the versatility of SIFT-MS and its ability to give real time data means it has been used for many varied applications. These include:

i. On-line real time quantification of multiple volatile breath metabolites for cohorts of healthy adults and children.[137,157–159]
ii. The comparative analyses of breath exhaled via the mouth and nose that identify systemic and orally-generated compounds.[151]
iii. The identification of volatile compounds released by malignant lung cells *in-vitro* and *in-vivo*.[160–162]
iv. The identification of volatile compounds in exhaled breath and urine headspace in patients with upper GI malignancy.[163,164]
v. The enhancement of breath metabolites by drug ingestion.[165]
vi. The emission of volatile compounds from urine (especially ketone bodies) and from skin.[166]
vii. Quantification of carbon dioxide in breath.[167]

1.4.5 Other analytical techniques for exhaled breath analysis

In addition to SIFT-MS there are a number of other analytical techniques for the analysis of exhaled breath. The characteristics of the most frequently used techniques are summarised in Table 1.

Table 1: Characteristics of different analytical techniques used for exhaled breath analysis (adapted from Amann *et al)*[168]

Technique	What it detects	Pros	Cons
GC-MS	Molecular fragments	Different substance in gas sample can be identified	Sample have to be collected in bags / traps Quantification can be difficult
PTR-MS	Protonated molecular species	Highly sensitive on-line measurement	Water content of samples can cause problems Identification of compounds from *m/z* alone is sometimes difficult
SIFT-MS	Variety of ionic species characteristic of precursor ions and reactant molecules	On-line, real-time and absolute quantification of several compounds simultaneously to good accuracy. Water vapour as internal calibration	Identification of isomers sometimes difficult
IMS	Protonated molecular species	Small, portable device with highly sensitivity	Quantification is difficult
Laser spectrometry	Characteristic spectral lines	Potentially small device, on-line measurement possible	Specific laser necessary for each molecular species. Water content of sample may cause problems

GC-MS: gas chromatography mass spectrometry, PTR-MS: proton transfer reaction mass spectrometry, SIFT-MS: Selection ion flow tube mass spectrometry, IMS: ion mobility spectrometry, *m/z*: mass-to-charge ratio.

1.5 OTHER POTENTIAL BIOMARKERS OF *PSEUDOMONAS AERUGINOSA* INFECTION

1.5.1 Introduction

Using a variety of methods, 60 volatile organic compounds have been identified as possible markers of PA (see Table 2). The vast majority of these do not have the appropriate specificity as they are also identified in the headspace of other bacterial cultures. In addition to HCN, the other compounds that appear to be specific to PA and therefore also have the potential to be used as diagnostic markers are 2-aminoacetophenone, 2-nonanone and methyl thiocyanate.

Table 2: Volatile organic compounds associated with *Pseudomonas aeruginosa* and their method of detection

	VOC	Detection methods						Reference
		GC-MS	GC-FID	SIFT-MS	PTR-MS	CI-MS	SESI-MS	
1	1-butanol	*		*	*	*	*	Mayr[169], Shestivska[170], Thorn[171], Zechman[172], Zhu[173]
2	1-pentanol						*	Thorn[171]
3	1-undecene	*	*					Scholler[174], Zechman[172]
4	2-aminoacetophenone	*	*	*			*	Cox[175], Labows[176], Scott-Thomas[177], Smith[178], Thorn[171], Zhu[173]
5	2-aminopyridine	*						Shestivska[170]
6	2-butanol	*						Zechman[172]
7	2-buanone	*						Shestivska[170]
8	2-heptanone	*						Zechman[172]
9	2-nonanone	*		*			*	Savalev[179], Smith[178], Zechman[172], Zhu[173],
10	2-pentanone						*	Zhu[173]
11	2-propanol	*						Shestivska[170]
12	2-undecanone	*		*				Smith[178], Zechman[172],
13	3-methyl-1-butanol	*		*				Labows[176]
14	3-penten-2-one	*						Shestivska[170]
15	3-penten-2-one-4-methyl	*						Shestivska[170]
16	4-methylphenol						*	Zhu[173]
17	Acetaldehyde	*		*				Shestivska[170]
18	Acetic Acid	*		*			*	Carroll[135], Shestivska[170], Zhu[173]

	VOC	Detection methods						Reference
		GC-MS	GC-FID	SIFT-MS	PTR-MS	CI-MS	SESI-MS	
19	Acetone	*		*			*	Carroll[135], Shestivska[170], Zhu[173]
20	Acetonitrile			*			*	Carroll [135], Zhu[173]
21	Acetophenone	*						Shestivska[170]
22	Ammonia	*		*				Shestivska[170], Thorn[171],
23	Butanal	*						Shestivska[170]
24	Butanal,2-methyl	*						Shestivska[170]
25	Butanal,3-methyl	*						Shestivska[170]
26	Butanol			*				Shestivska[170]
27	Butanone	*		*				Carroll[135], Zechman[172]
28	Butyric acid			*				Shestivska[170]
29	Carbon disulphide	*						Shestivska[170]
30	Diethyl ether			*				Shestivska[170]
31	Dimethyl disulphide	*	*	*	*			Carroll[135], Mayr[169], Scholler[174], Shestivska[170]
32	Dimethyl sulphide	*		*				Carroll[135], Shestivska[170]
33	Dimethyl trisulphide	*	*	*	*			Mayr[169], Scholler[174], Shestivska[170]
34	Ethanol	*		*			*	Carroll[135], Thorn[171], Zhu[173]
35	Ethyl butanoate							Thorn[171]
36	Ethylene glycol						*	Zhu[173]
37	Formaldehyde	*		*				Shestivska[170], Thorn[171]
38	Hexanoic acid			*				Shestivska[170]
39	Hydrogen cyanide			*				Carroll[135], Shestivska[170]
40	Hydrogen sulphide			*				Thorn[171]
41	Indole			*			*	Thorn[171], Zhu[173]
42	Isopentanol	*					*	Zechman[172], Zhu[173]
43	Isopentyl acetate	*						Zechman[172]
44	Isoprene	*	*	*				Carroll[135], Scholler[174], Thorn[171]
45	Metanethiol (methyl mercaptan)			*				Carroll[135], Shestivska[170], Thorn[171]
46	Methanol			*				Carroll[135], Shestivska[170]
47	Methyl phenol			*				Shestivska[170]
48	Methyl butanal	*						Zechman[172]
49	Methyl butenol	*						Zechman[172]
50	Methyl thiocyanate	*		*				Shestivska[170]
51	Methyl thiolacetate	*						Shestivska[170]
52	Nitric oxide			*				Enderby[136], Jaffe[180]
53	Pentanoic acid			*				Shestivska[170]
54	Pentanone	*						Zechman[172]
55	Phenol			*				Shestivska[170]
56	Propanol (1.2)			*				Shestivska[170]
57	Pyrimidine						*	Zhu[173]
58	Toluene	*		*	*			Labows[176], Mayr[169], Zechman[172]
59	Trimethylamine							Thorn[171]
60	Undecene	*						Zechman[172]

GC-MS: Gas chromatography mass spectrometry, GC-FID: Gas chromatography flame ionisation detector, SIFT-MS: Selected ion flow tube mass spectrometry, PTR-MS: Proton transfer reaction mass spectrometry, CI-MS: Chemical ionisation mass spectrometry, SESI-MS: secondary electrospray ionisation mass spectrometry.

1.5.2 2-aminoacetaphenone

The sweet “grape-like” odour sometimes associated with the growth of PA has been identified as 2-aminoacetaphenone (2-AA).[175] A recent study from New Zealand used GC-MS to identified 2-AA in the headspace of PA cultures[177]. 2-AA was also detected in the breath of 15/16 (94%) patients with chronic PA infection compared to 5/17 (29%) healthy controls and 4/13 (31%) CF patients free-from PA infection. This suggests that 2-AA may be a biomarker of PA infection but the high rate of false positives means that its specificity is poor.

1.5.3 Methyl thiocyanate

One of the ways in which PA detoxifies HCN is through its metabolism to methyl thiocyanate. Using SIFT-MS, Methyl thiocyanate at a concentration >6 ppbv has been identified in the headspace of 28/36 PA cultures.[170] All these cultures also produced HCN. Methyl thiocyanate was identified in the exhaled breath of 28 children with CF at a concentration of 2-21 ppbv but there was no significant difference between patients with and without PA infection.

1.5.4 2-nonanone

Using solid-phase microextraction (SPME) 2-nonanone has been measured in the headspace of sputum samples.[179] This was then compared to routine microbiology culture as a marker for the presence of PA in sputum. 2-nonanone is a marker for the presence of PA in sputum with a sensitivity of 72% and a specificity of 88%. The 2-nonanone results were available significantly quicker than the culture results. Further research needs to be undertaken to investigate if 2-nonanone is a breath marker of PA infection, this is difficult as it is non-volatile.

1.5.5 Summary of potential biomarker for *Pseudomonas aeruginosa*

A number of potential biomarkers have been associated with PA. The four that seem to be specific to PA are HCN, methyl thiocyanate, 2-AA and 2-nonanone. Of these; 2-nonanone is non-volatile and therefore is difficult to detect in exhaled breath, methyl thiocyanate did not does not differentiate between PA infected and non-PA infected CF patients and 2-AA has a high rate of false positives. This leads HCN as the most likely useful marker of PA infection.

1.6 SUMMARY

In summary cystic fibrosis is an inherited, life shortening disease that affects multiple organs. The respiratory component is characterised by recurrent infections and chronic inflammation. PA is one such infection and its detrimental effect on morbidity and mortality is well established. Early diagnosis is important but this is difficult in patients who cannot expectorate sputum. A breath test to diagnose PA is very appealing as it could be used in young children who often have the most difficulty in expectorating sputum. Initial research has suggested that HCN is a potential biomarker that could be used in a diagnostic breath test for PA. This thesis will attempt to analyse the factors that influence the *in-vitro* production of HCN by PA and then assess if exhaled breath HCN is a useful early marker of PA infection.

Chapter Two

Aims & Hypotheses

CHAPTER TWO – AIMS & HYPOTHESES

2.1 HYDROGEN CYANIDE AS AN *IN-VITRO* MARKER OF *PSEUDOMONAS AERUGINOSA*

The studies in this thesis will aim to assess and quantify the effect of genotype (strain), phenotype (mucoid / non-mucoid), culture conditions (biofilm / planktonic) and duration of incubation on the production of hydrogen cyanide (HCN) by Pseudomonas aeruginosa (PA).

- HYPOTHESIS 1: The quantity of HCN produced by PA cultures varies according to genotype (strain). [Investigated in Study 1]
- HYPOTHESIS 2: PA cultures with a mucoid phenotype produce more HCN than PA cultures with a non-mucoid phenotype. [Investigated in Study 1]
- HYPOTHESIS 3: PA samples cultured under biofilm conditions produce more HCN than PA samples cultured under planktonic conditions. [Investigated in Study 2]
- HYPOTHESIS 4: The quantity of HCN produced by PA cultures varies according to the duration of incubation. [Investigated in Studies 1 & 2]

2.2 HYDROGEN CYANIDE AS AN *IN-VIVO* MARKER OF *PSEUDOMONAS AERUGINOSA* INFECTION IN CHILDREN

The ultimate aim of this chapter is to assess if HCN is an early marker of PA infection in children with CF. The multi-centre cohort study (The SPACE Study) which will investigate this will collect breath samples from children with CF who are free from PA infection. Due to the numbers and geography involved in this study the samples will be collected in breath sampling bags and then transferred to the SIFT-MS instrument for off-line analysis. Prior to planning the final methodology

for the SPACE Study the type of breath sampling bag that provides the optimal conditions for breath samples containing HCN needs to be investigated, as does the maximum duration of sample storage prior to analysis and the optimal temperature for analysis.

- HYPOTHESIS 5: The duration a breath sample containing HCN can be stored prior to analysis is dependent on the types of breath sampling bag used. [Investigated in Study 3]
- HYPOTHESIS 6: Warming breath samples to body temperature prior to analysis improves the accuracy of the results. [Investigated in Study 3]
- HYPOTHESIS 7: HCN is a specific and sensitive marker of early PA infection in children with CF. [Investigated in Study 4]]

2.3 HYDROGEN CYANIDE AS AN *IN-VIVO* MARKER OF *PSEUDOMONAS AERUGINOSA* INFECTION IN ADULTS

To date, the vast majority of *in-vivo* studies involving HCN as a marker of PA have involved children with CF. This is because a percentage of healthy adults generate HCN in their oral cavity therefore any investigation of HCN as a marker of PA infection in CF adults would have to use nose-exhaled breath.

- HYPOTHESIS 8: Nose-exhaled HCN concentration is an in-vivo marker of chronic PA infection in adults. [Investigated in Study 5]

2.4 HYDROGEN CYANIDE AS A MARKER OF BURKHOLDERIA CEPACIA COMPLEX INFECTION

It was previously thought that PA was the only organism frequently found in the CF lung to produce HCN. A recent *in-vitro* study used a cyanide ion-selective electrode to demonstrate the production of cyanide by BCC when cultured under biofilm but not planktonic conditions. This *in-vitro* experiment is replicated using SIFT-MS to investigate is HCN is a marker of BCC infection. The exhaled breath HCN concentration of patients with chronic BCC infection is also analysed.

- HYPOTHESIS 9: HCN is an in-vitro marker of BCC when cultured under biofilm but not planktonic conditions [Investigated in Study 6]
- HYPOTHESIS 10: HCN is an in-vivo maker of BCC infection in those patients with biofilm BCC [Investigated in Study 6]

Chapter Three

Methodologies

CHAPTER THREE – METHODOLOGIES

3.1 INTRODUCTION

In this chapter contains the various methodologies used in the subsequent studies. The methods sections of the subsequent studies will then refer back to this chapter.

3.2 MICROBIOLOGY

3.2.1 *Pseudomonas aeruginosa* Cultures

The PA isolates used in the *in-vitro* studies were collected and stored as part of a previous study looking at cross infection between CF patients at the University Hospital of North Staffordshire[181]. The 98 isolates were obtained from CF patients between January and May 2007, genotyped and then stored on cryogenic beads (Pro-Lab Diagnostics. Microbank™ Product code PL.172) at -70 degrees Celsius in the microbiology laboratory.

3.2.1.1 Preparation of *Pseudomonas aeruginosa* agar plate cultures

New cultures were created by placing a single cryogenic bead directly onto a blood agar plate (Oxoid Ltd, Product code: CM0331) and plating it out. From this new plate out, as many new cultures were created as required from a single isolate. Each of the new plates were covered with a lid, sealed in an individual low-density polyethylene bags (127mm x 203mm) and incubated at 37^{o}C.

3.2.1.2 Preparation of *Pseudomonas aeruginosa* biofilm cultures

PA biofilm cultures were created by using one of the cryogenic beads to inoculate a 20ml Brain Heart Infusion (BHI) enrichment broth (Oxoid Ltd, Product Code CM1135), which was incubated in air at 37°C for 48 hours. 10ml of the inoculated BHI broth was then pipetted into a sterile 90mm Petri dish (volume 62ml) containing 35g of 4mm diameter sterile glass beads (sufficient to evenly cover the bottom of the Petri dish). A lid was then placed on the Petri dishes and the dish sealed in an individual low-density polyethylene bags (127mm x 203mm) and incubated at 37°C. The BHI broth media was changed daily by pipetting off approximately 10mls of spent media and replacing with 10mls of fresh BHI broth.

3.2.1.3 Preparation of *Pseudomonas aeruginosa* planktonic cultures

The methodology for preparing planktonic PA cultures was identical to that for biofilm cultures explained in section 3.2.1.2 except that the inoculated BHI broth was pipetted into a sterile 90mm Petri dish that does not contain any glass beads.

3.2.2 *Burkholderia Cepacia Complex* Cultures

Burkholderia cepacia complex (BCC) isolates were obtained from sputum samples collected from CF patients known to be chronically infected with BCC.

3.2.2.1 Preparation of *Burkholderia cepacia complex* agar plate cultures

Sputum samples were homogenised and cultured on a *B. cepacia* medium (Oxoid Ltd - Product code: PO0938) at 35-37^{o}C for 48 hours. If BCC was not isolated then the plates were cultured for a further 5 days at 28-30^{o}C. BCC colonies were confirmed visually. If there was any uncertainty about identification, further tests

were undertaken which included: biochemical analysis (enzymatic and carbohydrate assimilation tests), gram staining and PCR.

3.2.2.2 Preparation of *Burkholderia Cepacia Complex* biofilm cultures

One or 2 colonies of the BCC from the agar plate cultures were used to inoculate 20ml of BHI enrichment broth, which was incubated at 37^{o}C for 18 hours. Broths were sub-cultured to check purity. The BCC containing broths were serial diluted with sterile saline to achieve a turbidity of 0.5 optical density units measured by spectrophotometry (set at 600 nanometers [nm]). This approximately correlates to 10^{8} CFU/ml. BCC biofilm cultures were developed by pipetting 10ml of BCC inoculated BHI broth into a 90mm Petri dish containing 35g of 4.0mm diameter sterile glass beads. The Petri dish was then covered with a lid, placed in a sealed low-density polyethylene bags (127mm x 203mm) and incubated at 37°C. The BHI broth media was changed daily by pipetting off approximately 10mls of spent media and replacing with 10mls of fresh BHI broth.

3.2.2.3 Preparation of *Burkholderia Cepacia Complex* planktonic cultures

The methodology for preparing planktonic BCC cultures was identical to that for biofilm cultures explained in section 3.2.2.1 except that the inoculated BHI broth was pipetted into a sterile 90mm Petri dish that did not contain any glass beads.

3.2.3 Biofilm and Planktonic Control Cultures

PA and BCC biofilm and planktonic control cultures were created using exactly the same methodology except a sterile BHI broth was used

3.2.4 Confirmation of Biofilm Formation

Biofilm formation was assessed visually on a daily basis and quantitatively after 96 hours of incubation. For the quantitative assessment, 12 glass beads were removed from the centre of each biofilm culture and placed in the wells on a microtitre plate. The wells were washed three times with 200µl sterile water and then stained with 200µl of Crystal violet (1% weight/ volume (w/v)) for 15 minutes at 20^{o}C, allowing time for the crystal violet to penetrate the bacteria cell wall and membrane. Each well was washed a further 3 times with sterile water and allowed to air dry for 45 minutes. 200µl of industrial methylated spirits (IMS) (70% v/w) was then added to each well and incubated at 30°C for 15 minutes to solubilise the biofilm. 200µl of IMS (containing the solubilised biofilm) was then extracted from around the glass beads and pipetted into a well on a new microtitre plate. This was then analysed using a spectrophotometer set at 600 nm. As the number of bacteria in the biofilm increase, the aqueous concentration of crystal violet increases which causes increased absorbance. This method of assessing biofilm formation has previously been described[130] but a possible criticism of this method is that it is actually assessing bead adherence and not biofilm formation. True biofilm formation can be assessed by the detection of quorum sensing signalling molecules but we do not have this facility in our laboratory.[182]

3.2.5 Control Cultures

Control cultures of *Staphylococcus aureus, Streptococcus pneumonia* and *Moraxella catarrhalis* were created by inoculating a blood agar plate (Oxoid Ltd, Product code: CM0331) with stored clinical samples of each of these organisms. *Haemophilus influenzae* control cultures were created in the same way using

chocolate agar plates (Oxoid Ltd, Product code: CM0271) and *Aspergillus fumigatus* using Sabouraud agar (Oxoid Ltd, Product code: CM0041). The inoculated plate were then incubated at 37^{o}C for 48 hours.

3.2.6 Acknowledgments

I would like to acknowledge the help and support I received from Dr Alice Alcock and Hayley Sims in the preparation of the various cultures and the assessment of biofilm formation.

3.3 SELECTED ION FLOW TUBE MASS SPECTROMETRY (SIFT-MS)

3.3.1 Overview

SIFT-MS combines fast flow tube reactors and quantitative mass spectrometry to allow accurate real-time quantification of volatile compounds.[151,152] See Figure 7. It is this ability to give absolute quantification of a number of compounds simultaneously in real-time that makes SIFT-MS ideally suited to breath analysis. A mixture of precursor (reagent) ions is generated in a discharge ion source. The appropriate ion species (H_3O^+, NO^+ or O_2^+) is mass selected by a quadrupole mass filter, according to the compounds to be quantified, and then injected as selected ionic species into fast-flowing helium carrier gas in a flow tube. The ion species chosen for these experiments is H_3O^+ as it reacts rapidly with HCN, acetone, ammonia and ethanol[153]. The gas to be analysed (breath or culture headspace) is sampled directly into the carrier gas / precursor ion swarm at a known rate via a calibrated capillary. The reagent ions react with the trace gases in the sample producing ions that are characteristic of the trace gas molecules. These characteristic product ions, and the precursor ions and their hydrates are detected and counted by a downstream quadrupole mass spectrometer/ion

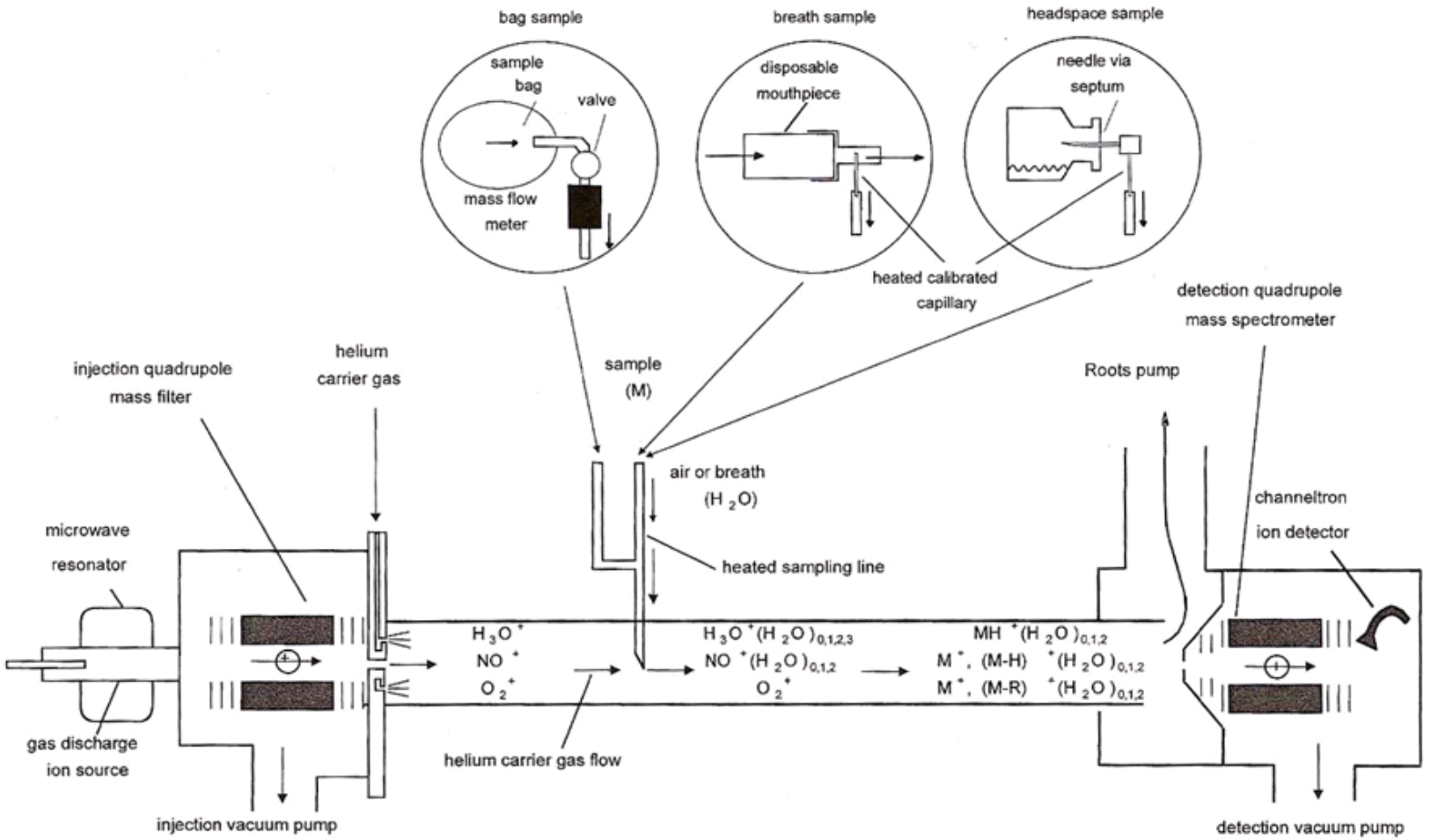
detection system. An on-line computer calculates the partial pressures of the trace gases in the air sample from the precursor ion and characteristic product ion count rates and the sample gas flow rate as established by the calibrated capillary.[183] A *Profile 3* SIFT-MS instrument (*Trans Spectra Limited*, UK) was used for these studies.

3.3.2 Modes of Operation

SIFT-MS instruments can be operated in ether the full scan (FS) or multiple ion monitoring (MIM) mode. Using the full-scan (FS) mode a complete mass spectrum is obtained by sweeping the detection quadrupole over a chosen mass-charge ratio (m/z) range. This is done for a selected time whilst a sample of air or breath is introduced into the carrier gas at a steady flow rate. An example is shown in Figure 3. The count rates of the ions are then calculated from the numbers of counts and the total sampling time for each ion. The mass spectra are interpreted by relating the product ion peaks to the trace gases present in the sample from a detailed knowledge of the ion chemistry and the in-built database. Using the MIM mode only the count rates of the precursor ions and those of selected product ions are monitored. This is achieved by rapidly switching the downstream mass spectrometer between the masses of all the primary ions and the selected product ions and dwelling on each of these masses for a predetermined short time interval. This real-time monitoring is possible because of the fast time response of SIFT-MS. This is about 20 milliseconds and is largely determined by the fast flow rates of the carrier gas along the flow tube and the sample gas along the inlet tube. There is no fundamental limit to the number of different ion masses that can be recorded simultaneously using this technique. However, with the values of the

precursor ion count rates currently available in SIFT-MS instruments, the practical limit is about 14 ions at trace gas concentrations in the ppb regime. If larger numbers of ions need to be monitored it is much more convenient to sequentially record several full scan spectra in the time allowed by the sample volume and construct a table of count rates of all ions within the m/z range.[154]

Figure 7: The SIFT-MS Instrument



Used with permission from Smith.[184]

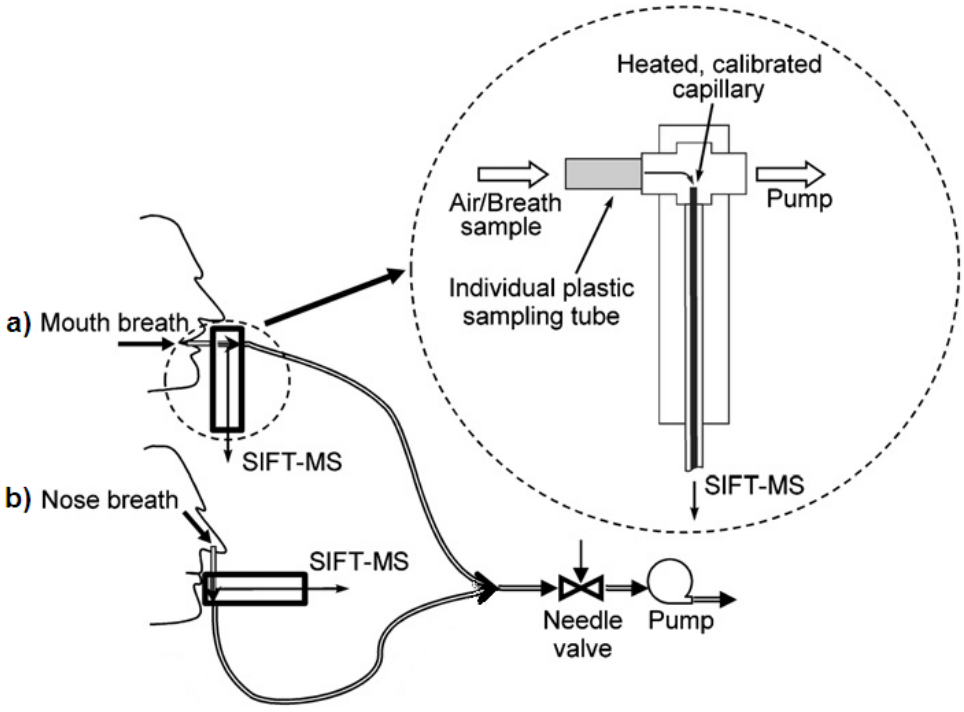
3.3.3 Analysis of Mouth and Nose-Exhaled Breath Samples

To enable simultaneous mouth and nose sampling, a small vacuum pump is included in the sampling line to draw breath across the entrance to the sampling capillary. See Figure 8. The flow rate of the breath sample is adjusted using the in-line needle valve to be much less than the breath exhalation rate, but greater that the flow rate through the capillary (also shown in Figure 8). This ensures that the normal breath exhalations from the mouth (a) and from the nose (b) are not compromised.

Mouth exhalations are provided through a mouthpiece attached to a bacterial filter and then to the sample inlet line on the SIFT-MS instrument. The subject is asked to provide three slow mouth exhalations in succession. Nose exhalations are made through a soft silicone tube attached to a bacterial filter and then to the sample inlet line of the SIFT-MS instrument. Whilst the silicone tube is held in one nostril, the subject is asked to provide three slow nose exhalations with the other nostril and mouth closed. The concentration of the relevant compounds is then measured in the end-tidal portion of each of the 3 breath exhalations and the average concentration calculated. The end-tidal portion is determined by the water vapour concentration that is simultaneously measured.[185] This is the standard method used for the measurement of volatile organic compounds in exhaled breath. When measuring biomarkers in relation to pulmonary infection there is logic to using the initial portion of the exhaled breath as infections largely reside in the connecting airways. In practice in makes very little difference to the mean concentrations which portion of the exhaled breath is used.

Figure 8: Mouth and nose-exhaled breath sampling

A representation of the sampling of a) breath exhaled via the mouth and b) breath exhaled via the nose. The breath samples are drawn along the individual plastic sampling tube and across the sampling calibrated capillary (shown in enlargement) by the action of a small pump. The in-line needle valve is used to regulate the breath flow rate across the sampling capillary.



3.3.4 Collection of Breath Samples in Sampling Bags

Breath samples are collected through a mouthpiece attached to the bag. All the bags used had a volume of 1000ml and are filled fully prior to analysis. When possible, this is done using a single slow exhalation, but younger children may require more than one breath. When the bag is inflated the mouthpiece should be removed and the bag sealed.

3.3.5 Analysis of Exhaled Breath Samples Collected in Sampling Bags

Off-line analysis refers to the analysis of breath samples that have previously been collected in sampling bags. The commercially manufactured Tedlar bags are connected to the sample inlet arm of the SIFT-MS instrument via the valve on the bag. The disposable Nalophan bags made in our laboratory from sheet material, are punctured with a hypodermic needle attached to the sample inlet arm. The mean concentration of water vapour and any other selected volatile compounds is determined over 100 seconds whilst operating the instrument in the multiple ion monitoring mode.

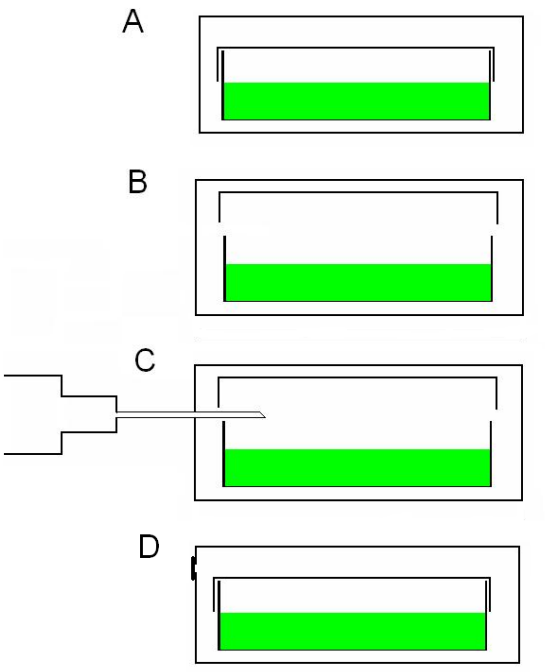
3.3.6 Analysis of Culture Headspace

As previously detailed all cultures are prepared in Petri dishes, covered with a lid and sealed inside individual low-density polyethylene bags (127mm x 203mm). At the time of analysis, the lid on the Petri is lifted whilst keeping the bag sealed. The bag is then punctured with a hypodermic needle connected directly to the sample inlet line of the SIFT-MS instrument and the needle placed under the lid in the culture headspace. See Figure 9 A, B, C.

The gas / vapour developed above the cultures is introduced at a flow rate of 24 mL/min (via a heated calibrated capillary) into the carrier gas of the SIFT-MS instrument. The "Multi Ion Monitoring" (MIM) mode of operation of SIFT-MS[152] is used to focus on and to analyse the compounds of interest in the samples. The sample is analysed for 100 seconds and the mean concentrations of the relevant compounds are recorded over this time. Following analysis, the hypodermic needle is removed whilst keeping the bag sealed, the lid replaced and the puncture hole left by the needle covered with tape. See Figure 9 D. This technique allows the culture headspace to be analysed at multiple durations of incubation.

Figure 9: Analysis of culture headspace

A: Petri dish culture with lid in sealed bag. B: Lid lifted whilst keeping bag sealed. C: Bag pierced with hypodermic needle attached to sample inlet line of SIFT-MS instrument and needle held in headspace for analysis. D: Needle removed, lid replaced and puncture hole covered with tape.



Chapter Four

Hydrogen cyanide as an *in-vitro* marker of *Pseudomonas aeruginosa*

CHAPTER FOUR – HYDROGEN CYANIDE AS AN *IN-VITRO* MARKER OF *PSEUDOMONAS AERUGINOSA*

4.1 STUDY ONE: DOES THE *PSEUDOMONAS AERUGINOSA* GENOTYPE OR PHENOTYPE AFFECT HYDROGEN CYANIDE PRODUCTION?

4.1.1 Introduction

This study will investigate if the quantity of HCN produced by cultures of PA is repeatable and if production by PA is affected by the PA genotype or phenotype (mucoid or non-mucoid).

4.1.1.1 Genotype

Quorum sensing is the process by which organisms use extracellular molecules to regulate phenotypes in response to population density. The ability of PA to employ quorum sensing and the subsequent relationship between cyanide concentration and bacterial load, supports the hypothesis that the observed variation in HCN production is dependent on PA strain. Cyanide production by PA is regulated in part by quorum sensing, with high population densities inducing synthesis.[143–145] Despite this, cyanide concentrations (measured using a cyanide ion-selective electrode) are independent of PA bacterial load when measured in the sputum of CF and non-CF bronchiectasis patients, suggesting cyanide and HCN production is dependent on the presence of specific PA strains (genotypes).[133] This is also supported by the finding that certain epidemic strains of PA overproduce specific quorum sensing regulated exoproducts.[146] This is the first reported study to assess the variation in cyanide production by different PA strains.

4.1.1.2 Phenotype

The acquisition of the *mucA* mutation leads to the non-regulation of alginate production and the development of a mucoid phenotype when grown *in vitro*. This is associated with increased antibiotic resistance, the formation of biofilms and the development of chronic infection.[80] Previous studies have shown that a gene involved in the regulation of alginate synthesis (AlgR) acts as an activator of the genes that regulated HCN production (*hcnABC*) in mucoid PA strains and a suppressor in non-mucoid PA strains.[186] The hypothesis that is therefore proposed is that mucoid PA strains will produce more HCN than non-mucoid strains:

4.1.2 Methods:

4.1.2.1 Experimental design

As part of a previous study looking at cross infection between CF patients, all PA isolates obtained from CF patients between January and May 2007 were genotyped[181]. The 98 PA isolates were stored on cryogenic beads at -70 degrees Celsius in our microbiology laboratory. For this study, 8 new agar plate cultures were prepared from each of the 98 original isolates. This was done using the methodology described in 3.2.1.1. The HCN concentration of the culture headspace of 2 of these samples (referred to hereafter as paired PA cultures) were analysed after 24 hours incubation, a further 2 after 48 hours incubation, a further 2 after 72 hours incubation and the final 2 after 96 hours incubation. Previous studies had analysed HCN production up to 48 hours but concentrations were still rising at this point,[135] we therefore decided to extend the period of analysis to 96 hours. This analysis was done using the methodology described in

3.3.6. The repeatability of the HCN generation by PA was assessed by comparing the HCN produced by the paired PA cultures.

Control cultures of *Streptococcus pneumoniae, Staphylococcus aureus, Moraxella catarrhalis, Haemophilus influenzae* and *Aspergillus fumigatus* were created using the methodology in 3.2.5. From each control culture, eight agar plates were plated out and incubated in sealed low-density polyethylene bags. These organisms were chosen as they are the commonest organisms (in addition to PA) that are found in the CF lung. The culture headspace was analysed after 24, 48, 72 and 96 hours using the methodology described in 3.3.6. Chocolate agar plates (Oxoid Ltd, Product code: CM0271) were used for the *Haemophilus influenzae* samples, Sabouraud agar (Oxoid Ltd, Product code: CM0041) for the *Aspergillus fumigatus* samples and blood agar (Oxoid Ltd, Product code: CM0331) for the others. The headspace of five sterile blood agar plates and five sterile chocolate agar plates incubated in sealed low-density polyethylene bags were analysed after the same durations.

4.1.2.2 Statistical methods

SPSS Statistics Version 21 (IBM Corp. Released 2012) was used for the analysis. In order to assess the reliability of the process the intraclass correlation (ICC) was calculated using a two way ANOVA with random effects. This assesses how strongly units in the same group resemble each other. As the raw data was heavily skewed and remained non-normal even after a log transformation, the 95% confidence interval (CI) for the ICC was generated using 1000 bootstrapped samples. This is a way of assigning measures of accuracy. The Shapiro Wilks test confirmed that the differences remained non-normal even after transformation

of the original values. Consequently median differences between the paired samples at each of the four time points were generated with corresponding 95% CI. In this way the magnitude of differences between the paired samples at each of the time points could be assessed.

The HCN concentration for the 96 pairs of PA cultures was then averaged at each time point. A repeated measures MANOVA (Wilks' Lamda) was used to compare the overall distribution of HCN production between phenotypes. A two way MANOVA was also adopted to assess the effect of the three commonest strains and phenotype across all time points. In both cases a log transformation was used to ensure homogeneity of covariance matrices across the groups. Scheffé's *post hoc test* was then performed to identify differences between the mucoid and non-mucoid groups at each time point and similarly between the three strains. These analyses were repeated on the ranked data as a form of sensitivity analysis.

4.1.3 Results

4.1.3.1 Background data

Of the 98 genotyped PA samples that had been stored as part of the previous study, 96 were available for analysis. Forty eight samples were mucoid and 48 were non-mucoid. There were 26 different strains (genotypes), 10 of which were epidemic strains. Four of these had been previously described: Liverpool (n=8), Midlands_1 (n=19), Midlands_2 (n=4) and Stoke (n=15) and six had not: Epidemic_1 (n=4), Epidemic_2 (n=5), Epidemic_3 (n=2), Epidemic_4 (n=3), Epidemic_5 (n=3) and Epidemic_6 (n=2). The remaining 16 genotypes were unique to individual patients; 10 were isolated on more than one occasion:

Sporadic_1 (n=5), Sporadic_2 (n=3), Sporadic_3 (n=3), Sporadic_4 (n=2), Sporadic_5 (n=2), Sporadic_6 (n=2), Sporadic_7 (n=2), Sporadic_8 (n=2), Sporadic_9 (n=2) and Sporadic_10 (n=2) and six on a single occasion: Sporadic_11-16.

4.1.3.2 Reproducibility of hydrogen cyanide generation by paired *Pseudomonas aeruginosa* cultures

The reproducibility of HCN generation by the paired PA cultures was analysed by calculating the intraclass correlation and the median difference in HCN concentration. As can be seen in Table 3 the intraclass correlation was high and the median difference low at all time points. This suggests that HCN production by cultures of PA is repeatable.

Table 3: The reproducibility of hydrogen cyanide generation by paired Pseudomonas aeruginosa cultures

Incubation Period	Intraclass correlation	Median difference in HCN concentration (95% C.I)
24 hours	0.97 (0.96, 0.98)	3 (-1 to 6)
48 hours	0.97 (0.96, 0.98)	3 (1 to 8)
72 hours	0.97 (0.96, 0.98)	9 (-1 to 27)
96 hours	0.96 (0.95, 0.98)	5.5 (-7 to 29)

HCN: hydrogen cyanide, C.I: confidence interval.

4.1.3.3 Headspace hydrogen cyanide concentrations for *Pseudomonas aeruginosa* and control cultures

The incubated blank plates and the cultures of *Streptococcus pneumoniae, Staphylococcus aureus, Moraxella catarrhalis, Haemophilus influenzae* and *Aspergillus fumigatus* produced <10ppbv of HCN at all time points. As expected the HCN was significantly higher in the headspace above the PA cultures (Table 4).

Table 4: Headspace hydrogen cyanide concentrations for *Pseudomonas aeruginosa* cultures, control cultures and incubated blank plates.

Agar	Organism	No of samples	HCN at 24 hours	HCN at 48 hours	HCN at 72 hours	HCN at 96 hours
Bl	PA	96	62 (17-188)	155 (30-1327)	743 (74-2670)	831 (89-2948)
Bl	SP	5	2 (2-2)	2 (1-2)	1 (1-2)	2 (1-3)
Bl	SA	5	1 (1-4)	4 (3-6)	3 (2-4)	2 (2-3)
Bl	MC	5	3 (2-6)	3 (2-5)	1 (1-3)	1 (0-2)
Ch	HI	5	2 (1-3)	2 (2-2)	6 (5-6)	2 (2-3)
Sab	AF	5	1(1-2)	3(2-3)	2(1-2)	1(1-1)
Bl	Blank	5	2 (1-2)	2 (1-4.5)	2 (2-2)	2 (2-3)
Ch	Blank	5	3 (3-4)	2 (2-2)	3 (2-3)	2 (1-2)
Sab	Blank	5	1(1-2)	2(2-3)	1(0-2)	3(2-4)

HCN: hydrogen cyanide, Bl: blood, Ch: chocolate, Sab: Sabouraud, PA: *Pseudomonas aeruginosa,* SP: *Streptococcus pneumoniae,* SA: *Staphylococcus aureus,* MC: *Moraxella catarrhalis,* HI: *Haemophilus influenzae,* AF: *Aspergillus fumigatus.* HCN presented as median (IQR) ppbv

4.1.3.5 Headspace hydrogen cyanide concentrations for different *Pseudomonas aeruginosa* strains

There are clear differences in the headspace HCN concentrations between different PA strains, although there is also variability within strains (Table 5). As genotype does affect HCN production, Hypothesis 1 is accepted.

Table 5: Headspace hydrogen cyanide concentrations for different *Pseudomonas aeruginosa* strains

Strain	No.of samples	HCN at 24 hours	HCN at 48 hours	HCN at 72 hours	HCN at 96 hours
Liverpool	8	92 (24-150)	525 (157-1397)	2238 (483-3682)	3093 (2495-4501)
Midlands_1	19	85 (55-158)	164 (90-815)	1451 (380-2634)	748 (550-2338)
Midlands_2	4	24 (14-266)	62 (22-1261)	199 (71-1371)	2186 (222-4471)
Stoke	15	16 (10-24)	24 (14-32)	37 (25-181)	75 (28-109)
Epidemic_1	4	499 (153-849)	6570 (4281-8033)	5852 (4858-7699)	8727 (7505-8978)
Epidemic_2	5	190 (78-306)	1833 (1020-3175)	3241 (1500-7122)	4336 (803-7239)
Epidemic_3	2	48 (33-63)	24 (15-32)	173 (100-245)	54 (35-73)
Epidemic_4	3	169 (145-174)	776 (626-1029)	7475 (7126-9128)	7483 (7382-8256)
Epidemic_5	3	13 (12-15)	22 (21-29)	39 (26-44)	89 (61-97)
Epidemic_6	2	194 (105-283)	1084 (564-1605)	1979 (1025-2933)	3527 (1826-5227)
Sporadic_1	5	21 (18-70)	37 (35-124)	46 (34-1274)	154 (62-2301)
Sporadic_2	3	16 (12-35)	27 (15-27)	167 (87-252)	588 (297-741)
Sporadic_3	3	272 (141-307)	1065 (620-1372)	3754 (2087-3965)	1989 (1558-2063)
Sporadic_4	2	16 (15-17)	74 (57-91)	126 (123-129)	62 (47-76)
Sporadic_5	2	22 (17-26)	15 (9-21)	6 (5-7)	10 (9-10)
Sporadic_6	2	1797 (967-2627)	4874 (2725-7024)	5871 (5311-6431)	6432 (5926-6939)
Sporadic_7	2	6406 (4231-8581)	8299 (7460-9139)	9174 (9012-9336)	7453 (7124-7781)
Sporadic_8	2	979 (853-1105)	5361 (3680-7052)	6965 (4928-9001)	4432 (2499-6364)
Sporadic_9	2	45 (34-56)	117 (90-143)	424 (255-593)	789 (684-895)
Sporadic_10	2	2716 (1389-4043)	1037 (830-1244)	8342 (7819-8864)	15714 (14630-16799)
Sporadic_11	1	946	1285	3505	3585
Sporadic_12	1	186	499	7559	12325
Sporadic_13	1	1002	5715	7621	5771
Sporadic_14	1	113	1127	16612	11923
Sporadic_15	1	738	2774	6861	12231
Sporadic_16	1	7	31	75	34

HCN: hydrogen cyanide, No: number. All values in ppbv, presented as median (IQR)

4.1.3.4 Headspace hydrogen cyanide concentration for mucoid and non-mucoid phenotypes

The comparison of the headspace HCN concentrations between the mucoid and non-mucoid phenotypes suggested increased production by the non-mucoid samples (Table 6). This difference was significant when analysed across all time points (Wilks' Lambda: $F_{4,91}$=4.35, p=0.003) and after 24 hours only (p=0.008) when the time points were analysed individually. The same conclusions were reached using the ranked data although the corresponding p-values were 0.018 and 0.007 respectively. As PA phenotype does affect HCN production Hypothesis 2 is accepted.

Table 6: Headspace hydrogen cyanide concentrations for mucoid and non-mucoid *Pseudomonas aeruginosa* samples

Phenotype	No. samples	HCN at 24 hours	HCN at 48 hours	HCN at 72 hours	HCN at 96 hours
Mucoid	48	43 (12-136)	127 (35-418)	525 (84-2234)	747 (91-3107)
Non-mucoid	48	99 (22-350)	626 (27-2279)	2196 (36-5528)	1441 (78-6429)

HCN (hydrogen cyanide) values in ppbv, presented as median (IQR)

4.1.3.5 Headspace hydrogen cyanide concentrations analysed for genotype and phenotype

Interestingly when headspace HCN concentrations were split according to genotype and phenotype (see Table 7), some strains had higher HCN for non-mucoid samples (Liverpool and Stoke) and some for mucoid samples (Midlands_1). Multivariate analysis of the effect of strain, phenotype and the interaction of both on the headspace HCN concentration above the three commonest strains (Liverpool, Midlands_2 and Stoke) was undertaken. This

showed a significant effect of strain (Wilks' Lambda: $F_{8,66}$=5.76, p<0.001), no effect from phenotype (Wilks' Lambda: $F_{4,33}$ =0.78, p=0.55) and a borderline interaction of strain and phenotype (Wilks' Lambda: $F_{8,66}$ = 2.01, p=0.051). The lack of effect of phenotype is unsurprising as the Liverpool and Stoke strains had higher HCN concentrations above non-mucoid samples, whereas the Midlands_1 strain had higher concentrations above mucoid samples. Further analysis revealed the headspace HCN concentration above the Stoke strain to be significantly lower than the other two strains at all four time points.

Table 7: Headspace hydrogen cyanide concentrations for mucoid and non-mucoid samples of the Liverpool, Midland_1 and Stoke strains

Strain	Phenotype	No. of samples	HCN at 24 hours	HCN at 48 hours	HCN at 72 hours	HCN at 96 hours
Liverpool	Mucoid	3	12 (11-70)	161 (156-526)	532 (435-988)	2860 (2131-2971)
Liverpool	Non-mucoid	5	123 (62-216)	949 (159-2740)	3341 (3034-4505)	4028 (3104-5921)
Midlands_1	Mucoid	14	127 (71-184)	182 (138-368)	1598 (816-2753)	804 (725-2393)
Midlands_1	Non-mucoid	5	58 (17-85)	87 (16-1240)	167 (29-1079)	544 (68-2183)
Stoke	Mucoid	7	11 (8-17)	10 (9-28)	56 (16-186)	39 (28-97)
Stoke	Non-mucoid	8	22 (15-29)	27 (23-33)	36 (33-104)	77 (48-119)

HCN (hydrogen cyanide) values in ppbv, presented as median (IQR)

4.1.4 Discussion

Study 1 is the first study to investigate the variation in HCN production between different strains of PA. This is important as previous studies have been unable to detect HCN or cyanide in a proportion of PA samples, raising the possibility that cyanogenesis does vary according to PA strain.[135,187] We have identified HCN in the headspace of all the PA samples analysed, reflecting the sensitivity of the SIFT-MS instrument. Despite all the samples producing HCN, there are clear

differences in the quantity produced by different PA strains. The low levels of HCN produced by the control cultures supports previous studies showing PA is one of a limited number of organisms to produce HCN. Previously the proposed cut-off for the HCN detected in the headspace over a culture to confirm PA was 100ppbv (sensitivity 68% and specificity 100%).[135] These data suggest that for the cultures included in this study, using the highest HCN concentration at any of the time points, the cut-off could be reduced to 10ppbv (sensitivity 100%, specificity 100%).

The very high correlation between the HCN produced by the pairs of PA cultures confirms the reproducibility of cyanide production by PA isolates and of the SIFT-MS real time analyses. Although there is some variation in the HCN production by samples of the same strain, overall there are clear differences between PA strains. This is the first study to show this difference. This study has also shown that all the PA samples produced detectable levels of HCN which reflects the high sensitivity and reliability of the SIFT-MS analyses. It is unclear how in-vitro HCN production by PA cultures correlates with in-vivo HCN detection in patient's breath or sputum. Specifically, *in-vivo* HCN levels tend to be lower,[133,134,136] although it is not known if current methods are sensitive enough to detect HCN in sputum or exhaled breath produced by a PA strain with lower cyanide production. It is also possible that the same strain of PA could produce different amounts of HCN depending on its position in the respiratory tract and the specific environmental conditions present.

Regarding phenotype, previous studies have reported higher cyanide production by mucoid PA cultures.[150,187] Our data suggest the opposite with non-mucoid

cultures having higher HCN concentrations. This may be related to the timing of analysis as the trend for HCN was still rising at 96 hours for the mucoid samples, whereas the non-mucoid samples peaked at 72 hours and started to fall at 96 hours. This study may therefore have observed a different effect of phenotype if HCN analysis was undertaken after a longer period of incubation. Interestingly when the HCN concentrations were analysed for strain and phenotype (Table 6) the effect of phenotype seemed to differ between strains. The observed difference in the effect of phenotype on cyanogenesis between this and other studies may therefore be explained by which PA strain were included in the previous studies.

Also relevant is the methodology used; detecting the non-volatile ion cyanide is very different to using SIFT-MS to detect the volatile ion HCN. A cyanide ion-selective micro-electrode is used to detect the cyanide trapped in a layer of sodium hydroxide. This process is far more disruptive to the culture than the analysis of the culture headspace. This raises the possibility that the mucoid PA cultures are producing more cyanide but the layer of alginate is preventing the release of HCN. As any *in-vivo* breath test would have to be based on the detection of a volatile ion, the factors that affect HCN and not cyanide production are most relevant. As early PA infection usually has a non-mucoid phenotype, higher HCN production by non-mucoid PA is an advantage for a breath test trying to detect early PA infection.

In conclusion, Study 1 has demonstrated that all of the PA samples in this study produced HCN but the quantity varied according to genotype and phenotype. This will supports the possible clinical applications of the cyanogenic properties of PA.

4.2 STUDY TWO – DOES THE FORMATION OF BIOFILMS OR CULTURE DURATION AFFECT HYDROGEN CYANIDE PRODUCTION BY *PSEUDOMOMONAS AERUGINOSA*?

4.2.1 Introduction

This study will investigate if the formation of biofilms or the duration of culture affects HCN production by PA.

4.2.1.1 Biofilm formation

During early infection, PA multiplies whilst in a planktonic (free-floating) form; it later forms biofilms which are non-motile communities embedded in an exopolysaccharide matrix attached to a solid surface.[98] The change from planktonic to biofilm growth is signalled by quorum sensing in which extra-cellular molecules regulate phenotype in response to population density.[143] As cyanide is known to be a quorum sensing molecule, the hypothesis proposed is that HCN production by PA will increase when it is cultured under biofilm conditions.[144] This hypothesis is supported by a previous study that found BCC produced cyanide when cultured under biofilm but not planktonic conditions.[130]

4.2.1.2 Culture duration

The concentration of any bacterial biomarker is dependent to some degree on the bacterial load. As the duration of culture incubation affects bacterial load, the hypothesis proposed is that this also affects HCN production:

4.2.2 Methods

4.2.2.1 Experimental design

For this study, 12 of the PA isolates from Study One were used. The 12 samples included 4 Liverpool Epidemic Strain, 4 Midlands_1 Strain and 4 Stoke Strain samples. The 4 samples of each genotype included 2 mucoid and 2 non-mucoid samples. The variation in headspace HCN concentrations seen in Study 1 informed the choice of sample size for this study. For each of the PA isolates, one biofilm PA culture was created using the methodology described in 3.2.1.2 and one planktonic PA culture was created using the methodology described in 3.2.1.3. The headspace HCN concentration of both culture types was analysed after 24, 48, 72 and 96 hours of incubation using the methodology described in 3.3.6. Two biofilm control cultures and two planktonic control cultures were created using the methodology described in 3.2.3 and their headspace HCN concentrations analysed as for the PA cultures.

4.2.2.2 Statistical methods

SPSS Statistics Version 21 (IBM Corp. Released 2012) was used for the analysis. The HCN concentrations were non-normally distributed and the results are therefore expressed as median (IQR). Mann-Whitney tests[188] were used in two group comparisons and robust confidence intervals generated for the Hodges-Lehmann median difference.[189] General Linear Modelling was applied to assess the effect of genotype, phenotype, culture duration and culture conditions. Log transformed data were used for this procedure in order to ensure homogeneity of variances. A p value <0.05 was deemed significant.

4.2.3 Results

All PA samples produced readily detectable concentrations of HCN; median concentration 144 (61-512) ppbv of headspace sample gas (see Table 8). The headspace HCN concentration of the 4 control cultures was <3 ppbv at all time points. When the HCN results were analysed according to phenotype, non-mucoid samples tended to produce higher concentrations of HCN than the mucoid samples (see Table 9). This was statistically significant at 24 hours (p=0.01) as highlighted by the 95% confidence interval for the difference in medians.

Table 8: Headspace hydrogen cyanide concentrations analysed according to genotype.

Culture Duration	Liverpool Strain (n=8)	Midlands_1 Strain (n=8)	Stoke Strain (n=8)
24 Hours	78 (47-522)	614 (396-942)	54 (37-91)
48 Hours	468 (129-733)	242 (201-411)	150 (98-296)
72 Hours	307 (97-1064)	115 (86-271)	210 (111-292)
96 Hours	728 (32-1429)	46 (22-239)	65 (41-108)

All data presented in ppbv as median (IQR).

Table 9: Headspace hydrogen cyanide concentrations analysed according to phenotype.

Culture Duration	Mucoid samples (n=12)	Non mucoid samples (n=12)	95% CI for Median Difference
24 Hours	51 (30-171)	531 (93-942)*	-370 (-814,-19)
48 Hours	241 (123-410)	241 (182-428)	-44 (-250, 153)
72 Hours	123 (94-293)	201 (88-586)	-36 (-391, 75)
96 Hours	40 (29-89)	117 (40-1373)	-59 (-1306, 10)

CI: confidence interval. All data presented in ppbv as median (IQR),
*p=0.01 as determined by Mann-Whitney test

Although there was a trend for biofilm samples to have higher headspace HCN concentrations than planktonic samples, this did not reach statistical significance at any time point (see Table 10).

Table 10: Headspace hydrogen cyanide concentrations analysed according to culture conditions.

Culture Duration	Biofilm samples (n=12)	Planktonic samples (n=12)	95% CI for Median Difference
24 Hours	198 (39-739)	87 (48-476)	24 (-64, 500)
48 Hours	292 (175-428)	216 (123-489)	34 (-215, 221)
72 Hours	205 (68-348)	127 (106-350)	-26 (-114, 197)
96 Hours	74 (32-379)	48 (34-269)	9.6 (-61, 168)

CI: confidence interval. All data presented in ppbv as median (IQR).

Multivariate analysis (General Linear Modelling) was used to assess the effect of culture duration, genotype, phenotype, culture conditions and their interactions. This showed a significant effect of culture duration ($p=0.005$), genotype ($p=0.0014$), phenotype ($p<0.001$) and the interactions between genotype/phenotype ($p<0.001$) and genotype/culture duration ($p=0.009$). A three way interaction between phenotype/genotype/culture duration was also observed ($p=0.009$). As culture conditions do not affect HCN production Hypothesis 3 is rejected. As culture duration does affect HCN production, Hypothesis 4 is accepted. The effect of culture conditions and the other interactions were not significant. This effect of culture duration, genotype and phenotype is in keeping with data from Study 1.

4.2.4 Discussion

This study confirms the effects of genotype and phenotype on HCN production by PA that were demonstrated in Study 1. In addition, this study investigates the effect of culture conditions (planktonic / biofilm) and duration of incubation on HCN production by PA. This study confirms that HCN production is affected by culture duration although the peak headspace HCN concentration is achieved at different durations of incubation for the different PA isolates. This HCN peak is likely to correlate with the peak in numbers of live PA bacteria. This in turn will be dependent on the exact bacterial mass at time 0, the rate of PA replication, the amount of substrate present and the rate of substrate use.

Although there was a trend for biofilm PA cultures to produce more HCN than planktonic PA cultures, this did not reach statistical significance. The insignificant difference in the amount of HCN produced by biofilm and planktonic PA samples therefore differs from BCC which has previously been shown to only produce cyanide when cultured under biofilm conditions.[130] One possible explanation for this is that cyanide is produced as a quorum sensing molecule but it remains trapped in the biofilm and is not released as HCN into the culture headspace. Any increased cyanide production would therefore go undetected using the methodology in this study.

Chapter Five

Hydrogen cyanide as an *in-vivo* marker of *Pseudomonas aeruginosa* in children

CHAPTER FIVE – HYDROGEN CYANIDE AS AN *IN-VIVO* MARKER OF *PSEUDOMONAS AERUGINOSA* IN CHILDREN

5.1 STUDY THREE: AN INVESTIGATION OF SUITABLE BAG MATERIALS FOR THE COLLECTION AND STORAGE OF BREATH SAMPLES CONTAINING HYDROGEN CYANIDE

5.1.1 Introduction

The research question that this chapter aims to answer in the chapter is: Can exhaled breath HCN be used as an early marker of PA infection in children with CF? This question will ultimately be addressed by Study Four - The Sensitivity and specificity of PA detection using the hydrogen Cyanide concentration of Exhaled breath (SPACE) Study. This is a 2 year observational study in which serial breath HCN concentrations are measured in 233 children with CF, free from PA. In this study, breath samples are collected in breath sampling bags and transferred to the SIFT-MS instrument for analysis. Previous research has been undertaken on the suitability of bags for breath sampling but not for samples containing HCN.[190–193] To effectively plan the methodology of Study Four, there needs to be an investigation of the type of breath sampling bag provides the optimal conditions for breath samples containing HCN, the maximum duration of storage and the optimal temperature for analysis.

5.1.2 Methods

5.1.2.1 Experimental design

15 children with CF were recruited (8 male, 7 female). Their median (IQR) age was 10 (9-13.5) years. Eight had chronic PA infection and 3 had CF related

diabetes did not. On a single visit, subjects provided an on-line mouth-exhaled breath sample using the methodology described in 3.3.3 and also gave breath samples into two 1000ml Tedlar sampling bags, two 1000ml 25 micron thick Nalophan (Nalophan 25) sampling bags and two 1000ml 70micron thick Nalophan (Nalophan 70) sampling bags in the methodology described in 3.3.4. The Tedlar bags were commercially manufactured (Cole-Parmer UK, Product number: GD0707-7000) and the Nalophan bags were made in our laboratory from sheet material.

One of each of the gas sampling bags was stored at room temperature (20^{o}C) and one in an incubator at body temperature (37^{o}C). Off-line analysis of the bag samples was undertaken after 1, 6, 24 and 48 hours of storage using the methodology described in 3.3.5. The timings of the analysis were informed by previous studies of stored breath samples which suggested that storing samples for longer than 1-2 days significantly affected the concentration of breath volatiles.[193] The on-line concentrations were used as a baseline and then compared to the various off-line concentrations. For both the on-line and off-line analysis HCN was measured in addition to acetone and water vapour. Water vapour acts as an internal control and its concentration in exhaled breath is well understood. Acetone is a systemic compound that is present in the exhaled breath of all individuals and, unlike HCN, is not generated to a significant extent in the mouth or airways. The concentration of acetone in exhaled breath is well known as are the factor which can cause this to rise or fall. It is therefore useful to monitor this metabolite in all breath analysis studies and especially in studies that compare on-line and off-line measurements.[194]

5.1.2.2 Statistics

SPSS Statistics Version 21 (IBM Corp. Released 2012) was used for the analysis. The concentrations of the acetone, HCN and water vapour were non-normally distributed, even after logarithmic transformation. Therefore, the results are displayed as median (IQR) values. The coefficient of determination was used to assess the correlation between on-line and off-line concentrations. The Mann-Whitney U test was used to assess the significance of the differences of concentrations between groups. A p-value of <0.05 was deemed significant. Uncertainty in the measurement of concentration was characterised by the standard error which was determined from the total number of ion counts recorded for each individual measurement.[183] It was thus found that for off-line measurements of HCN this uncertainty was within +/- 10% or +/- 1ppbv (whichever was greater) and for on-line HCN measurements it was within +/- 4ppbv. For the off-line measurements of acetone the uncertainty was always better than +/- 3.5% and for the on-line acetone measurements it was within +/- 12%.

5.1.2.3 Ethical approval

Ethical approval was granted by the Staffordshire Research and Ethics committee (05/Q2604/8). See Annex 2: 11.2.1.

5.1.3 Results

5.1.3.1 On-line concentrations

The median (IQR) concentrations of acetone was 311 (270.5-433) ppbv and HCN was 8.9 (4.4-13.7) ppbv. The median (IQR) on-line acetone concentration was significantly higher in those with CF-related diabetes compared to those without:

798 (652-1155) ppbv versus 303 (240-344) ppbv, p=0.02. The median (IQR) on-line HCN concentrations were significantly higher in those with chronic PA infection compared to those without chronic PA infection: 13.7 (11.3-15.5) ppbv versus 4.2 (2.5-4.6) ppbv, p=0.001. The median (IQR) on-line water vapour concentration was 5 (4.9-5.2)%, which is within the expected range for exhaled breath and the median (IQR) water vapour concentration of the laboratory air was 1.0 (0.9-1.1)%.

5.1.3.2 Off-line concentrations at 20^{o}C

Table 11 presents the results for breath samples contained in the bags formed from Tedlar and the two thicknesses of Nalophan that were analysed from 1 to 48 hours storage at room temperature (20^{o}C). Compared with the on-line HCN concentrations, the off-line HCN concentration were lower in all 3 types of bag at 1 hour, although correlation between off-line and on-line HCN was good. By 6 hours, although the correlation remained good, HCN concentrations in Nalophan 25 bags had fallen to approximately 30% of the on-line concentration and to 50% and 60% in Nalophan 70 and Tedlar bags, respectively. By 24 hours, the correlation between on-line and off-line HCN concentrations was poor.

Table 11: Off-line analysis of 3 compounds in breath samples stored at room temperature in bags made from different materials.

Storage time		Nalophan 25			Nalophan 70			Tedlar		
		HCN (ppbv)	**Acetone (ppbv)**	**Water (%)**	**HCN**	**Acetone**	**Water**	**HCN**	**Acetone**	**Water**
1 hr	Conc	6.5 (2.9-9.8)	247 (224-345)	2.2 (2.2-2.3)	5.9 (3.3-10.0)	259 (230-429)	2.3 (2.3-2.4)	6.7 (2.4-8.7)	262 (206-416)	3.3 (3.2-3.3)
	R^2	0.88	0.93	0.003	0.92	0.95	0.005	0.9	0.98	0.008
6 hrs	Conc	3.0 (1.4-4.0)	197 (167-317)	1.4 (1.3-1.4)	4.5 (2.0-8.0)	221 (178-388)	1.5 (1.4-1.5)	5.3 (1.9-7.3)	227 (186-369)	1.8 (1.7-1.9)
	R^2	0.68	0.88	0.003	0.8	0.89	0.02	0.84	0.92	0.03
24 hrs	Conc	1.2 (0.6-1.9)	111 (83-139)	1.1 (1.1-1.2)	2.2 (0.7-2.8)	130 (121-189)	1.1 (1-1.1)	2.6 (2.0-3.2)	132 (122-147)	1.2 (1.2-1.3)
	R^2	0.27	0.68	0.13	0.44	0.62	0.19	0.58	0.77	0.004
48 hrs	Conc	0.5 (0.4-0.6)	32 (27-35)	1.1 (1.0-1.1)	0.5 (0.3-1.0)	34 (29-37)	1.0 (1.0-1.0)	1.0 (0.9-1.2)	67 (63-75)	1.0 (1.0-1.1)
	R^2	0.15	0.08	0.11	0.26	0.27	0.53	0.12	0.54	0.001

HCN: hydrogen cyanide, conc: concentration, ppbv: parts per billion by volume, R^2: coefficient of determination. All concentrations presented as median(IQR).
Note: Median(IQR) on-line concentrations: HCN: 8.9(4.4-13.7) ppbv; acetone: 311(270.5-433) ppbv and water vapour: 5.0(4.9-5.2)%.

As expected, acetone was present in the exhaled breath at much higher concentrations than HCN. Correlation with on-line concentrations was good at 6 hours, but then declined. The water vapour concentration fell quickly with storage and by 24 hours it was similar to the atmospheric concentration. The correlation between on-line and off-line water vapour concentrations was poor, for all the bags at all the time points.

5.1.3.3 Off-line concentrations at 37^{o}C

Table 12 presents the results for samples from gas sampling bags analysed from 1 to 48 hours storage at body temperature (37^{o}C). The off-line HCN concentration fell with increasing durations of storage. For breath samples stored in Nalophan 25 bags the correlation between on-line and off-line HCN concentrations remained good up to 6 hours. At this point the HCN concentration had fallen to approximately 55% of the on-line concentration. For samples stored in Nalophan 70 and Tedlar bags the correlation between on-line and off-line HCN concentrations remained good up to 24 hours (see Figures 10 and 11). At this point the HCN concentration had fallen to approximately 60% and 65% of the on-line concentration, respectively. Again, acetone was present at a much higher concentration. Correlation with on-line concentrations was good up to 24 hours, but then declined. As in the samples stored at 20^{o}C, the water vapour concentration fell quickly with storage and by 24 hours it was similar to the atmospheric concentration. The correlation between on-line and off-line water vapour concentrations was also poor, for all the bags at all the time points. As warming the breath sample improved the correlation with the on-line sample, Hypothesis 6 is accepted.

Table 12: Off-line analysis of 3 compounds in breath samples stored at body temperature in bags made from different materials.

Storage time		Nalophan 25			Nalophan 70			Tedlar		
		HCN (ppbv)	Acetone (ppbv)	Water (%)	HCN (ppbv)	Acetone (ppbv)	Water (%)	HCN (ppbv)	Acetone (ppbv)	Water (%)
1 hr	Conc	7.0 (3.3-8.9)	267 (178-335)	2.4 (2.4-2.5)	7.1 (3.1-11.9)	271 (200-328)	2.6 (2.6-2.7)	7.8 (4.7-11.2)	273 (218-413)	3.6 (3.6-3.6)
	R^2	0.94	0.97	0.1	0.92	0.96	0.1	0.79	0.93	0.02
6 hrs	Conc	4.8 (1.8-7.3)	240 (162-295)	2.3 (2.3-2.4)	7.1 (2.5-9.7)	239 (150-322)	2.3 (2.3-2.4)	7.5 (3.3-7.9)	235 (173-362)	2.5 (2.4-2.5)
	R^2	0.79	0.93	0.02	0.92	0.94	0.06	0.92	0.97	0.06
24 hrs	Conc	2.1 (1.2-2.5)	199 (128-207)	1.7 (1.7-1.7)	5.4 (1.6-7.1)	209 (136-280)	1.8 (1.8-1.8)	5.8 (2.0-6.7)	201 (174-363)	2.0 (2.0-2.1)
	R^2	0.65	0.89	0.02	0.82	0.93	0.2	0.86	0.97	0.1
48 hrs	Conc	0.5 (0.4-0.5)	87 (67-94)	1.0 (1.0-1.0)	0.4 (0.4-0.5)	75 (59-83)	1.0 (1.0-1.0)	0.4 (0.3-0.4)	56 (49-68)	1.2 (1.2-1.2)
	R^2	0.03	0.64	0.04	0.18	0.47	0.01	0.04	0.69	0.4

HCN: hydrogen cyanide, conc: concentration, ppbv: parts per billion by volume, R^2: coefficient of determination. All concentrations presented as median(IQR).
Note: Median (IQR) on-line concentrations: HCN: 8.9 (4.4-13.7) ppbv; acetone: 311 (270.5-433) ppbv and water vapour: 5.0(4.9-5.2)%.

Figure 10: Correlation of online and offline hydrogen cyanide concentrations for samples stored in Nalophan 70 bags at 37°C for 24 hours

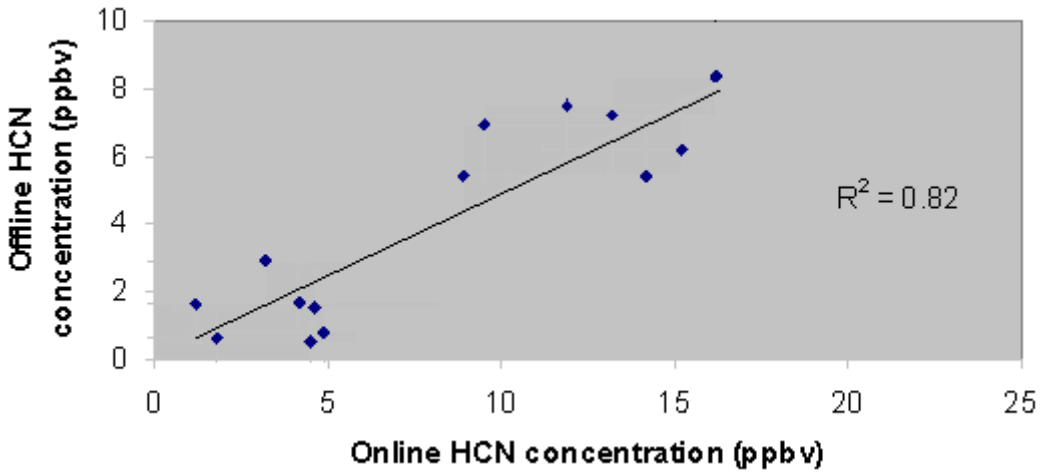
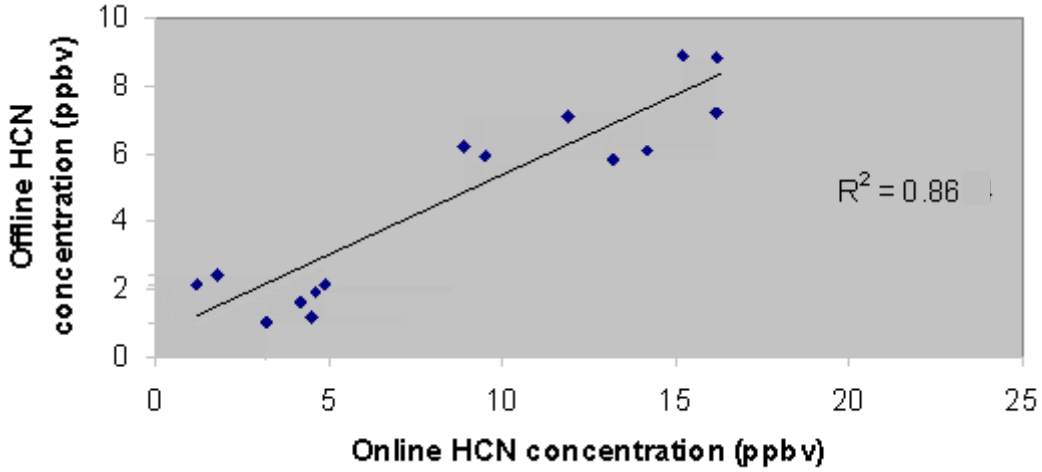


Figure 11: Correlation of online and offline hydrogen cyanide concentrations for samples stored in Tedlar bags at 37°C for 24 hours



5.1.3.4 Off-line hydrogen cyanide concentrations in patients with and without *Pseudomonas aeruginosa* infection

Table 13 compares the HCN concentration of breath samples of 8 children with and 7 without chronic PA infection, stored up to 24h. As with the on-line HCN

concentrations the off-line HCN concentrations (up to 24 hours) are significantly higher in those with chronic PA infection compared to those without. Figures 10 and 11 demonstrate the good separation of HCN concentrations for children with chronic PA infection and those without, for breath samples stored in Nalophan 70 and Tedlar bags for 24 hours. A threshold HCN concentration of approximately 5ppbv in breath samples stored in Nalophan 70 or Tedlar bags for up to 24 hours seems to provide good separation between the PA infected and non-PA infected children. The varying performance of the different types of breath sampling bags means Hypothesis 5 is accepted.

5.1.3.5 Analysis of samples stored at room temperature and then warmed prior to analysis

To make the logistics of the SPACE study easier, we wanted to investigate if storing the sample at room temperature and then warming prior to analysis gave similar results as keeping the sample at room temperature. On a different day, five of the same volunteers provided an online breath sample and a sample into a Nalophan 70 bag. The bag sample was stored at room temperature for 24 hours and then warmed to 37^{o}C prior to analysis. The median (IQR) online concentrations were: acetone 320 (275-431); HCN 8.4 (5.0-12.8) and water 4.9 (4.8-5.1) ppbv. At 24 hours the median (IQR) off-line concentrations were: acetone 2.1 (154-292), R^2=0.94; HCN 5.1 (1.8-7.8), R^2=0.80 and water 1.6 (1.6-1.6), R^2=0.10. These results suggest that storing the sample at room temperature and warming the sample prior to analysis gives similar results to those when the sample is keep warm throughout.

Table 13: Off-line hydrogen cyanide concentrations in breath samples stored at 37^{o}C for patients with and without chronic *Pseudomonas aeruginosa* infection

		1 Hour			6 Hours			24 Hours		
		Nalophan 25	**Nalophan 70**	**Tedlar**	**Nalophan 25**	**Nalophan 70**	**Tedlar**	**Nalophan 25**	**Nalophan 70**	**Tedlar**
PA	Median	10.5	11.9	11.4	7.3	9.7	8.3	2.5	7.1	6.7
	IQR	8.2-12.4	10.0-13.2	10.0-12.4	6.6-9.0	8.0-11.3	7.7-8.9	2.3-3.0	6.0-7.7	6.1-7.6
	Range	7.0-13.5	7.1-14.0	7.8-14.1	4.8-12.1	6.9-12.5	7.5-10.1	2.1-3.3	5.4-8.4	5.8-8.8
No PA	Median	3.2	3.0	4.1	1.5	2.4	3.5	1.1	1.5	1.9
	IQR	3.0-3.6	2.7-3.3	3.7-5.4	1.2-3.0	1.8-2.5	3.1-3.6	1.0-1.5	0.7-1.7	1.4-2.1
	Range	2.6-4.5	2.0-4.1	3.5-5.6	0.8-4.3	1.7-3.6	2.4-4.8	0.3-1.9	0.6-2.9	1.0-2.4
PA v No PA	p value	0.001	0.001	0.001	0.008	0.001	0.001	0.001	0.001	0.001

PA*: Pseudomonas aeruginosa.* All results are in parts per billion by volume, ppbv

5.1.4 Discussion

To our knowledge this is the first study to investigate the correlation between on-line and off-line HCN and acetone concentrations measured by SIFT-MS. As expected, the off-line concentrations were lower than on-line concentrations and the off-line concentrations fell further with increasing storage time. Warming the breath samples to body temperature decreased the rate at which the off-line concentrations fell and improved the correlation between off-line and on-line concentrations. The Nalophan 70 bag consistently performed better than the Nalophan 25 and almost to the level of the Tedlar bag. In breath samples stored in a Nalophan 70 or Tedlar bag at 37^{o}C, the off-line acetone and HCN concentrations correlated well with on-line concentrations for up to 24 hours of storage. The off-line HCN concentrations insamples stored at 37^{o}C were significantly higher in those patients with chronic PA infection compared to those without PA infection. A threshold HCN concentration of around 5ppbv would give good separation between patients with and without PA infection for samples stored up to 24 hours in Nalophan 70 or Tedlar bags.

The immediate difference in on-line and off-line concentrations can be explained by the different breath sampling techniques used for the measurements. During on-line analysis, the SIFT-MS instrument gives the concentration of the selected volatile compounds throughout the whole breath, in real time. The operator can then use the water vapour concentration that is simultaneously measured, to select the alveolar portion of the breath and report the volatile compound concentration during this section of the breath. In contrast, when breath samples are given into sampling bags the sample contains a mixed expiratory sample,

including both the alveolar portion of the breath and air from the rest of the bronchial tree. This results in a dilution of the alveolar portion of the breath and a lowering of the concentration of measured volatile compounds.

The fall in off-line concentrations with increasing durations of storage has been well described and is thought to be due to a combination of diffusion of compounds through the bag wall, adsorption and condensation.[190,195] As the temperature of breath sample falls below 37^{o}C, water vapour condenses on the inside of the bag and takes down water soluble compounds. By warming the sample to body temperature, any gases or vapours adsorbed onto the bag surface or water which have condensated can return to the gaseous state. These processes explain why the off-line concentrations decreased at a slower rate and correlated better with on-line concentrations when the samples were stored at body temperature. The diffusion of compounds through the wall of gas sampling bags has also been well described.[195] As this rate of diffusion will be slower in thicker wall bags this explains the difference in the fall in concentrations between the Nalophan 25 and Nalophan 70 bags.

A previous study compared different sampling containers (including Tedlar bags) for the sampling and storage of 6 volatile sulphur compounds relevant to breath analysis.[193] They demonstrated that samples stored in Tedlar showed losses of <10% when stored for 6-8 hours. When the dilutional difference between the on-line and off-line samples is taken into consideration, our results are similar to the results of this study. For the purpose of the SPACE study (Study 4) we are able to tolerate some HCN losses, providing the correlation between on-line and off-line

concentrations is good and allows differentiation between those patients with PA infection and those without.

In all 3 types of collection bag the correlation of off-line and on-line acetone concentrations was better than that for on-line and off-line HCN concentrations. This is likely to be related to acetone being less water soluble than HCN resulting in decreased losses through condensation and the greater diffusion rate of the lighter molecules through the bag material. The rapid fall in the water concentration of the breath samples down to the ambient air levels by 24 hours is similar to previous studies[195] and suggests that diffusion is the major cause of the falling concentrations. Both Tedlar and Nalophan are known to be permeable to water; this is a benificial characteristic of Nalophan which is used in food packaging.

The good correlation of on-line and off-line HCN measurements in the Nalophan 70 and Tedlar bag and the finding that a single HCN concentration can be used as a threshold for PA diagnosis for up to 24 hours breath sample storage time, suggests that either would be appropriate to use for the SPACE study (Study 4). As the Nalophan 70 bags are much cheaper than the Tedlar bags it means that they can be discarded after a single use, removing the need for bag cleaning or infection control measures. For The SPACE study, Nalophan 70 bags were therefore selected. Using the Nalophan 70 bags we demonstrated that keeping the sample at room temperature for 24 hours and warming the sample prior to analysis produces similar results as when the sample is kept warm for the duration. We will therefore transport samples at room temperature for the SPACE study and warm them prior to analysis.

5.1.5 Funding

We gratefully acknowledge funding for Study 3 by the University Hospital of North Staffordshire, Guy Hilton Research Trust (R5240A988).

5.2 STUDY FOUR: THE SENSITIVITY AND SPECIFICITY OF *PSEUDOMONAS AERUGINOSA* DETECTION USING THE HYDROGEN CYANIDE CONCENTRATION OF EXHALED BREATH - THE SPACE STUDY

5.2.1 Introduction

The ultimate aim of the research into HCN and PA is to investigate if HCN detection could be used in an exhaled breath test for the diagnosis of PA. This could potentially be of huge benefit to those children with CF in whom the diagnosis of PA is difficult, specifically those who are unable or unwilling to expectorate sputum. To investigate if HCN can be used in this way, breath samples need to be collected around the time CF children acquire PA infection. As you cannot predict when children with CF will acquire PA infection, regular breath samples need to be collected from a large cohort of CF children free from PA infection. A percentage of these children will acquire PA infection during the study period and the HCN concentration of the breath samples taken around the time of the PA isolate can then be examined to see if they indicated infection. The SPACE study was therefore designed to investigate if HCN is an early marker of PA infection in children with CF.

5.2.2 Methods

A cohort of children with CF who were free from PA infection was followed for approximately two years. At each routine CF appointment a breath sample was collected for HCN analysis and a clinical details questionnaire was completed. At the end of the study period the results of the microbiology samples taken as part of normal CF care were revealed to the study team and compared to the exhaled breath HCN concentrations.

5.2.2.1 Sample size and recruiting centres

The power calculation was based on the expected rise in exhaled breath HCN concentration in those who acquired a new PA infection[136]. Aiming for a sensitivity and specificity of 95% it was estimated that 46 new PA cases were needed. A retrospective audit at one of the recruiting centres found the rate of new PA infections to be 0.1 cases per patient year. The total patient years required to originate 46 new PA was therefore estimated to be 460, which was achieved by following 230 patients for 2 years. It was initially thought that this number of children could be recruited from seven Paediatric centres across the Midlands and North West of the UK. Due to fewer children meeting the inclusion criteria at these centres than was initially estimated, recruitment started at an eighth centre (Centre 1) approximately six months later.

5.2.2.2 Inclusion criteria

The inclusion criteria for children were:

- A diagnosis of CF confirmed by sweat test or genotyping
- 2-16 years of age
- Not to have isolated PA for the previous 12 months
- Have all their follow-up at centres included in the SPACE study (most shared-care patients were therefore not eligible).
- Be able to blow in to a sampling bag (hence the minimum age)

Informed consent and when appropriate informed assent was obtained for all recruited children.

5.2.2.3 Exhaled breath sample collection

The methodology for the breath sample collection and analysis was defined by Study Three, specifically it informed the maximum storage time prior to analysis and the need for pre-warming prior to analysis. Breath samples were collected by the child blowing into a 1000ml 70 micron nalophan bag as described in 3.3.4. It was requested that the child had not eaten or drunk anything for 30 minutes prior to providing the sample. Once the sample was collected the mouth piece was removed and the bag sealed. The bag was then transported to the SIFT-MS instrument and the HCN, acetone and ammonia concentration analysed using the methodology described in 3.3.5. This analysis had to be undertaken within 24 hours of the breath sample being taken. Prior to analysis the sample was warmed to 37^{o}C. These results were recorded on a secure database. A nalophan bag filled from a cylinder of medical air (BOC order code: 191-E) was analysed as a control every month.

5.2.2.4 Clinical details questionnaire

At each visit a clinical details questionnaire was completed. This data was collected to assess if children were symptomatic at the time of PA infection. See Annex 1: 11.1. This collected data on the child's general state of health (very well, well, okay, not as well as normal or unwell), any change in specific symptoms (cough, sputum production, shortness of breath and exercise tolerance) and antibiotic use (oral, nebulised and IV). These data were stored on a secure database.

5.2.2.5 Microbiology samples

During the study period a cough swab or sputum sample was taken at each out-patient appointment as part of the child's normal CF care. No microbiology samples were taken specifically for the SPACE Study or at the request of the SPACE Study team. The SPACE Study team were only informed of the microbiology results at the end of the study when all PA positive results were passed on to the Chief Investigator.

5.2.2.6 Ethical approval

Ethical approval for the SPACE Study was granted by the Coventry and Warwickshire Research and Ethics Committee (Ref: 10/H1211/48). Annex 2: 11.2.2.

5.2.2.7 Statistical analysis

SPSS Statistics Version 21 (IBM Corp. Released 2012) was used for the analysis. None of the data in this study was normally distributed and is therefore displayed as median (IQR). For the initial analysis, a breath HCN concentration of ≥5 ppbv was taken as a positive result (as suggested by Study 3). The ideal cut-off was confirmed by assessing the HCN concentration that produced the highest Youden Index. This is a measure of diagnostic accuracy: (sensitivity + specificity) -1. The Mann-Whitney U test was used to assess the significance of the differences between 2 groups and the Kruskal-Wallis test when there were ≥3 groups. The PA incidence rates were calculated by numerator / denominator. The numerator was the number of children with a new isolate of PA during the study period and the denominator was the 'person-time at risk'. The person-time at risk was the sum of the time from recruitment to new PA isolate for those that isolated PA and

the total follow-up time for those who did not isolate PA. The Poisson 95% confidence interval were calculated for the incidence rates and the Chi squared test was used to compare the significance of 2 incidence rates. A p-value of <0.05 was deemed significant.

5.2.3 Results

5.2.3.1 Recruitment and follow-up

A total of 233 children were recruited from the 8 centres. Their median (IQR) age was 8.0 (5.0-12.2) years. At each centre recruitment was open for 6 months but the start date varied between centres due to administrative reasons. The eighth centre (Centre 1) was added to ensure adequate numbers. It opened 6 months after the other centres had closed to recruitment. The first child was recruited in June 2011 and the last child in October 2012. The study closed at all centres in December 2013. The median duration of follow-up was 2.0 (1.7-2.3) years. Ten children were withdrawn from the study prior to the close date. Six transitioned to adult services and four moved area. The data on these ten children have been included in the analysis up to the point they were withdrawn.

5.2.3.2 *Pseudomonas aeruginosa* status

None of the children had isolated PA in the year prior to recruitment but they could be divided into three groups according to their PA status: 113 children had never grown PA (Never group), 99 children had previously isolated PA but were not currently receiving any nebulised anti-PA therapy (Free from [FF] group) and 21 were free from PA for > 1 year and were receiving prophylactic nebulised anti-PA therapy (Free from on treatment [FF-Tx] group). A summary of study data for the three groups is shown in Table 14. All those in the FF-Tx group had been

commenced on anti-PA therapy due to a previous diagnosis of chronic PA infection. The median age was highest for the FF-Tx group and lowest for the Never group.

As can be seen in Table 14, the use of oral antibiotics was very similar for the three groups (p=0.90), as was the use of IV antibiotics in the Never and FF groups (p=0.52). The median (IQR) number of IV antibiotic courses per patient year was higher in the FF-Tx group compared to the Never and FF groups combined: 1.1 (0-1.7) vs 0.0 (0-0.5), p<0.001. At recruitment the median (IQR) time since the last PA isolate was similar for the FF and the FF-Tx groups: 2.2 (1.6-4.7) vs 2.0 (1.7-2.9) years, p=0.29.

Table 14: Summary of study data according to *Pseudomonas aeruginosa* status

PA Status	No. children	Age [years]	Duration of follow-up [years]	Study activity			PA incidence (95% C.I) [cases per patient year]	Antibiotic courses	
				Total visits	Total breath samples	Samples per child		Oral [per patient year]	IV [per patient year]
Never	113	6.4 (4.0-10.9)	2.0 (1.7-2.3)	962	948	8 (6-11)	0.15 (0.10-0.22)	1.8 (0.7-3.1)	0 (0-0.5)
FF	99	8.1 (5.3-12.5)	2.1 (1.6-2.3)	910	898	9 (6-12)	0.19 (0.13-0.27)	1.8 (0.9-3.0)	0 (0-0.6)
FF Tx	21	13.1 (10.6-15.5)	2.0 (1.9-2.3)	214	209	10 (6-15)	0.41 (0.20-0.73)	1.8 (1.2-3.1)	1.1 (0-1.7)
Total	**233**	**8.0 (5.0-12.2)**	**2.0 (1.7-2.3)**	**2086**	**2055**	**9 (6-12)**	**0.19 (0.15-0.23)**	**1.8 (1.0-3.1)**	**0 (0-0.7)**

PA: *Pseudomonas aeruginosa*, IV: intravenous, FF: free from, FF-Tx: free from on treatment.

All data presented as median (IQR) apart from incidence rate which is presented as cases per patient year (95% confidence interval).

5.2.3.3 Study visits and breath samples

There were a total of 2086 study visits each with a completed clinical details questionnaire. Seven of the centres see the majority of their patients every 2 months although selected patients are seen less frequently. At Centre 4, all patients are seen less frequently although they request microbiology samples by post every month. This resulted in a lower median (IQR) number of study visits per patient year at Centre 4 compared to the other seven centres combined: 2.4 (1.9-2.8) vs 5.2 (4.6-6.3), $p<0.001$. A total of 2055 breath samples were analysed meaning there were 31 study visits without breath samples. Eight of these occurred as the child refused to provide a sample. For the remaining 23, samples were collected but they were not analysed within the 24 hour window due to problems with sample transport or the SIFT-MS instrument.

5.2.3.4 Incidence of new *Pseudomonas aeruginosa* isolates

In total, 71 children had a new isolate of PA during the study period (29 from the Never group, 31 from the FF group and 11 from the FF-Tx group). The median (IQR) age at recruitment was similar for those who subsequently grew PA and those who remained PA free: 7.7 (4.4-12.4) vs 8.2 (5.1-12.1) years, $p=0.71$. The overall incidence of PA isolates was 0.19 cases per patient year (95% confidence interval [CI]: 0.15-0.23). The incidence rate (95% CI) was similar in the Never and FF groups: 0.15 (0.10-0.22) vs 0.19 (0.13-0.27) cases per patient year, Chi squared 0.73, $p=0.39$). The incidence rate (95% CI) was higher in the FF-Tx group than in the Never and FF groups combined: 0.41 (0.20-0.73) vs 0.17 (0.14-0.22) cases per patient year, Chi squared 7.41, $p=0.007$). As can be seen in the Kaplan Meier curve in Figure 12 these incidence rates resulted in the proportion of

patients remaining free of PA at the end of the study period being 74% for the Never Group, 69% for the FF group and 48% for the FF-Tx group. For those that did isolate PA, the median time from recruitment to new PA isolate was 0.7 (0.3-1.3) years. This was similar for the never, FF and FF-Tx groups: 0.8 (0.3-1.3) vs 0.7 (0.3-1.4) vs 0.6 (0.3-1.0) years, p=0.89.

Table 15 shows a summary of the study data for the recruited children according to their CF centre. When calculating the PA incidence for each centre, children in the FF-Tx group were excluded. This is because the incidence of new PA isolates was higher in this group and its members (21 children) were not evenly distributed between the centres. Their inclusion would therefore have skewed the PA incidence. The overall PA incidence (95% C.I) for those children in the Never and FF groups was 0.17 (0.14-0.22) cases per patient year, this varied between 0.08 (0.04-0.18) and 0.28 (0.14-0.49) in the different centres. As can be seen in the Kaplan Meier curve in Figure 13 this resulted in the proportion of patients remaining free of PA at the end of the study varying between 56% (Centre 3) and 83% (Centre 7).

Figure 12: Kaplan Meier curve showing the proportion of children remaining free of *Pseudomonas aeruginosa* (PA) infection during the study period for each of the PA status groups

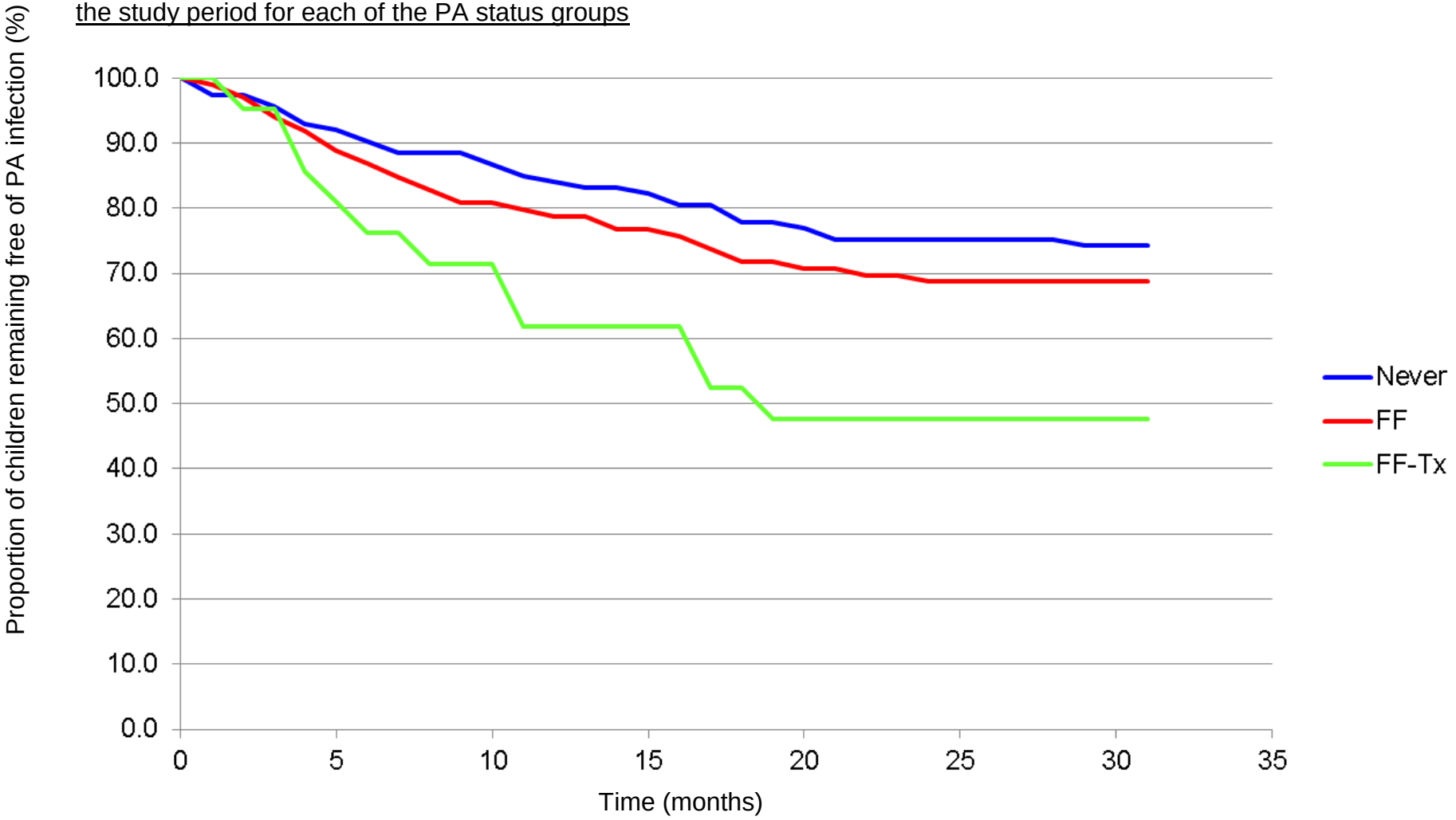


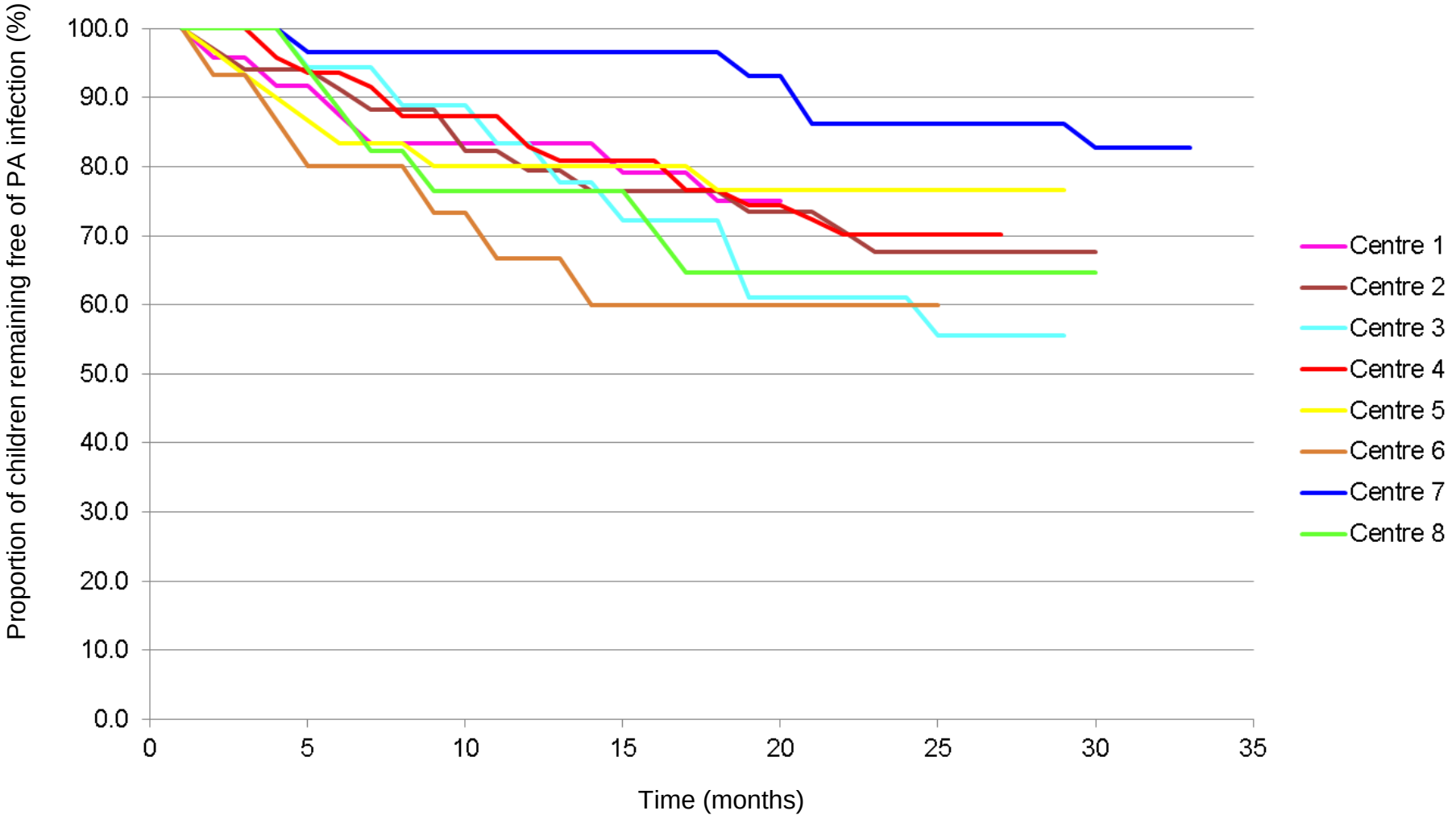
Table 15: Summary of study data according to recruiting centre

Centre	No. recruits	Age (years)	Duration of follow-up (years)	Study activity			PA incidence (95% C.I) [cases per patient year]	Antibiotic courses	
				Total visits	Total breath samples	Samples per child		Oral (per pt year)	IV (per pt year)
1	24	7.6 (4.0-8.0)	1.4 (1.3-1.4)	188	185	8.0 (6.8-9.0)	0.22 (0.10-0.40)	3.6 (3.1-4.2)	0 (0-0)
2	34	8.8 (6.0-12.0)	2.3 (2.2-2.3)	348	341	10.5 (10-12)	0.18 (0.10-0.29)	2.2 (1.7-2.7)	0 (0-0.4)
3	21	6.9 (4.9-13.2)	2.2 (2.2-2.3)	193	193	9 (8-10)	0.27 (0.15-0.43)	1.5 (0.6-2.2)	0(0-0)
4	53	9.1 (5.4-12.5)	1.8 (1.6-2.0)	229	220	5 (3-5)	0.20 (0.12-0.29)	0.6 (0.2-1.3)	0 (0-1)
5	30	9.4 (6.7-12.9)	2.2 (1.9-2.3)	306	305	11 (9-12)	0.13 (0.07-0.25)	1.5 (0.9-1.8)	0 (0-0.4)
6	18	8 (2.4-10.7)	1.8 (1.5-1.9)	136	133	7 (6-8)	0.28 (0.14-0.49)	2.6 (1.8-3.6)	0 (0-0.5)
7	36	5.2 (3.3-8.5)	2.3 (2.0-2.5)	477	474	14 (11-16)	0.08 (0.04-0.18)	2.9 (2.0-3.9)	0.4 (0-1.2)
8	17	8.4 (5.2-12.0)	2.4 (2.3-2.4)	209	204	12 (11-13)	0.22 (0.10-0.40)	2.6 (1.8-3.2)	0 (0-0)
Total	**233**	**8.0 (5.0-12.2)**	**2.0 (1.7-2.3)**	**2086**	**2055**	**9 (6-12)**	**0.17 (0.14-0.22)**	**1.8 (0.9-3.0)**	**0 (0-0.5)**

PA: *Pseudomonas aeruginosa*, IV: intravenous.

All data is presented as median (IQR) apart from incidence rate which is presented as cases per patient year (95% confidence interval)..

Figure 13: Kaplan Meier curve showing the proportion of children remaining free of *Pseudomonas aeruginosa* infection during the study period for each of the recruiting centres



For the same reason that children in the FF-Tx group were excluded from the PA incidence figures for individual centres, they were also excluded from the calculations of antibiotic courses per patient year for individual centres. See Table 15. Although the oral antibiotic use did not vary according to PA status, there was a wide variation between centres. The median (IQR) number of oral antibiotic courses per patient year varied between 0.6 (0.2-1.3) and 3.6 (3.1-4.2). The median (IQR) number of IV antibiotic courses at each of the centres varied between 0.0 (0-0) and 0.4 (0-1.2) courses per patient year.

5.2.3.5 *Pseudomonas aeruginosa* eradication

To avoid confusion when discussing PA eradication, the 11 children who isolated PA from the FF-Tx group have been excluded as they were already receiving nebulised anti-PA therapy. All of the 60 patients who isolated PA during the study period from the Never or FF groups received eradication therapy. Sixteen children received intravenous and nebulised antibiotics (six of these also received oral antibiotics). The remaining 44 received oral and nebulised antibiotics. A number of these patients were part of the TORPEDO-CF study which is investigating the best regimen for PA eradication. Fifty two of the 60 patients isolated PA early enough in the study to allow at least 6 months of follow-up and 44/52 (85%) were free of PA at this time point.

5.2.3.6 Pairing of study visits and *Pseudomonas aeruginosa* culture results

As mentioned above, 71 children had a new isolate of PA during the study period. For 57 of these children a study visit (with completed clinical details questionnaire and an analysed breath sample) had been undertaken on the date of the positive PA culture. These 57 questionnaires and breath samples are those used for the

analysis of symptoms and HCN concentration at the time of the PA positive result. Of the 14 new PA isolates without a paired study visit, eight were microbiology samples that were posted from home and did not coincide with an out-patient visit, four were microbiology samples that were obtained at hospital visits outside of a routine out-patient appointment and two occurred at an out-patient visit that the study team were unaware of due to the appointment having been rearranged.

5.2.3.7 Symptoms at the time of *Pseudomonas aeruginosa* isolation

At the time of PA isolation 24/57 (42%) children were asymptomatic. Of those that were symptomatic, cough was present in 30/33 (91%) of children, increased sputum in 16/33 (48%), increased shortness of breath in 9/33 (27%) and reduced exercise tolerance in 7/33 (21%). At the time of the PA isolate only 12/57 (21%) of children were described as “unwell” or “less well than normal”.

5.2.3.8 Breath hydrogen cyanide concentrations

There was no difference in the median (IQR) HCN concentration of the first breath samples for those in the Never, FF and FF-Tx groups: 2.2 (1.3-3.2) vs 2.3 (1.5-3.0) vs 2.4 (1.2-3.5) ppbv, p=0.58. For children that did not isolate PA during the study the median (IQR) HCN concentration of all their breath samples was 2.3 (1.4-3.1) ppbv. For the same children the median (IQR) difference between an individual’s maximum and minimum HCN concentration (intra-subject variation) throughout the study was: 2.8 (1.9-3.5) ppbv.

5.2.3.9 Elevated hydrogen cyanide concentrations in those who isolated *Pseudomonas aeruginosa*

Of the 57 children who had a new PA isolate with a paired study visit, 28 (49%) had a breath HCN concentration of >5ppbv at the time of the PA isolate. This

means that the sensitivity of PA detection using the HCN concentration of exhaled breath is 49%. Hypothesis 7 is therefore rejected. As expected, the median HCN concentration for these 28 children was significantly higher at the time of PA isolate compared to their first breath sample: 5.6 (5.4-5.9) vs 2.0 (1.3-3.1) ppbv, p<0.001. The remaining 29 children had a similar HCN concentration at the time of their PA isolate and on their first breath sample: 2.3 (1.8-3.0) vs 1.9 (1.0-2.7) ppbv, p=0.32. In 5 of the 28 children with elevated HCN, this had been elevated for 1 or 2 visits prior to the date of the PA isolate: median (IQR) duration 2.1 (1.7-2.5) months. All 28 patients with an elevated HCN stopped growing PA within 2 further study visits. In 20/28 children the HCN concentration was <5ppbv at the next study visit: median (IQR) HCN concentration was 2.5 (1.5-3.8) ppbv. In the remaining 8 children the median (IQR) duration that the HCN concentration remained >5ppbv: 2.3 (2.1-4.3) months.

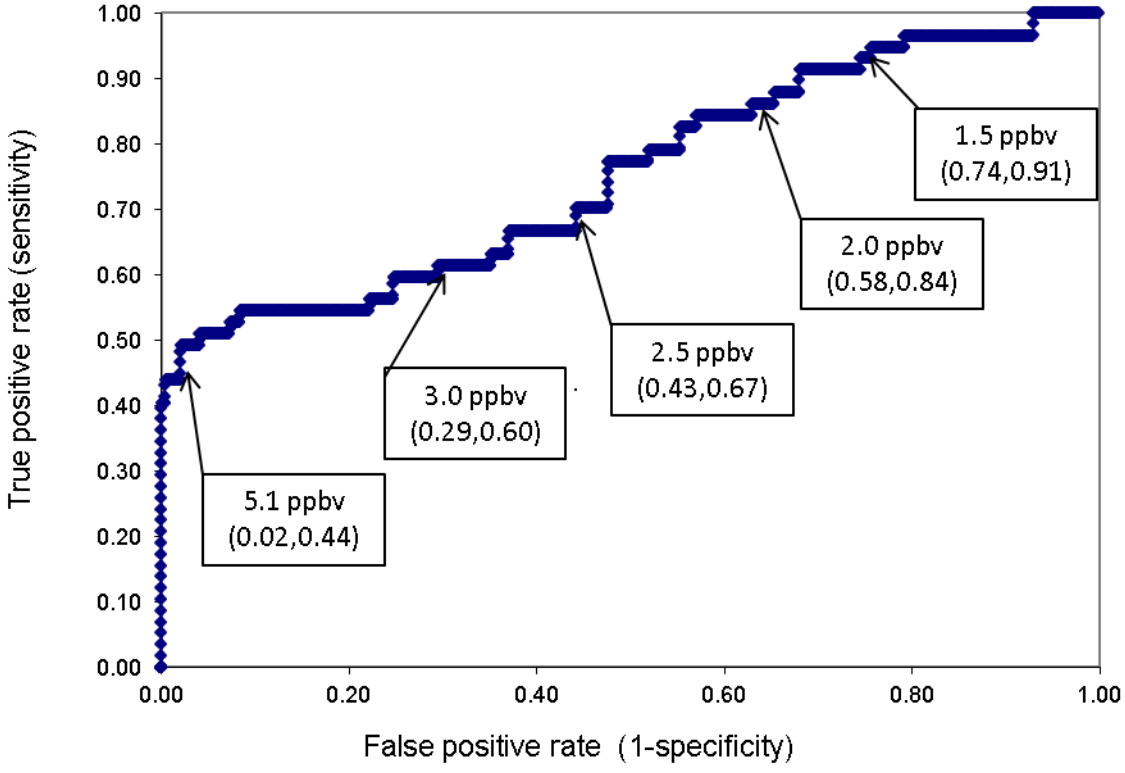
5.2.3.10 Elevated hydrogen cyanide concentrations in children who did not isolate *Pseudomonas aeruginosa*

Nineteen children who did not isolate PA had at least one breath sample with a HCN concentration >5ppbv. In eight of these children the elevation occurred on a single breath sample, in six children the HCN was elevated twice, in four it was elevated three times and in one it was elevated 4 times. The median HCN concentration of these 36 breath samples was 5.3 (5.1-5.7) ppbv. The number of false positives is 19 and the number of true negatives is 143 (number of children who did not isolate PA during the study and whose breath HCN concentrations always remained <5.0 ppbv). This means that the specificity of PA detection using the HCN concentration of exhaled breath is 88%. The positive predictive value is (28/47) 60% and the negative predictive value is (143/172) 83%.

5.2.3.11 Receiver operating characteristics (ROC) curve

The Receiver operating characteristics (ROC) curve generated from The SPACE Study data can be seen in Figure 14. The area under the curve is 0.75 which classifies the accuracy of exhaled breath HCN as a diagnostic test for PA infection as fair. The Youden Index is a measure of diagnostic accuracy: (sensitivity + specificity) -1. The maximum Youden index for these data is 0.47 when the HCN concentration is 5.1 ppbv. This supports this study's use of a cut-off for a positive result of 5 ppbv.

Figure 14: Receiver operating characteristics curve for The SPACE Study



5.2.3.12 Other *Pseudomonas* species

Apart from PA the only other species that has been shown to be cyanogenic is *Pseudomonas fluorescens* (PF).[80] Four patients isolated PF during the study

period. All the patients were asymptomatic at the time of the isolate and the children's HCN concentrations were not elevated at the time of the isolate.

5.2.3 Discussion

The SPACE study is the first *in-vivo* study to investigate if HCN in exhaled breath can be used as an early marker of PA infection in children with CF. As well as demonstrating that HCN is a specific marker of PA infection it has provided some very interesting prospective data on the incidence of PA infection in children with CF. The relatively low sensitivity means that at present it is not appropriate to use exhaled breath HCN as a screening test for PA infection. The low sensitivity is likely to be related to some PA isolates not producing enough HCN to allow detection in an exhaled breath test and the off-line methodology used to measure the HCN concentration in this study.

In Study 1, although all strains of PA had easily detectable concentrations of HCN in their culture headspace, the PA genotype did affect the quantity of detected HCN. A small number of strains produced relatively low headspace HCN concentrations with a >700 fold difference in the peak HCN concentration between the highest and lowest producing strains (15,714 v 22 ppbv). Although the exact correlation between the *in-vitro* headspace concentrations and the *in-vivo* exhaled breath HCN concentrations is unknown, it is reasonable to presume that pulmonary infection with one of the low HCN producing strains may not produce enough HCN to be detected on an exhaled breath test. The SPACE Study did not involve any analysis of the PA isolates so the PA genotype is unknown and no in-vivo analysis of HCN headspace production was made. The exact correlation between the HCN headspace concentration of an in-vitro culture and the exhaled

breath HCN concentration in a patient infected with the same organism is a possible area for future research. The inter and intra-subject variation of exhaled breath HCN concentrations is in keeping with previously pubished data and may be contributing to the low sensitivity.[136,196] It is known that dietary intake can affect the concentration of volatile organic compounds in exhaled breath. Although the children were requested not eat or drink anything for 30 minutes prior to providing the sample, this was not checked rigorously.

The SPACE study involved the analysis of breath samples taken at eight different CF centres across the Midlands and North West of England. As we only had one SIFT-MS instrument which is not easily transportable, the breath samples had to be collected in breath sampling bags and then transported to the SIFT-MS instrument for *off-line* analysis. Study 3 demonstrated that there is good correlation between the *on-line* and *off-line* HCN concentrations but the off-line concentrations were lower which could potentially affect the sensitivity. Analysis of the HCN concentrations of those children who isolated PA but did not have a breath HCN of >5ppbv showed it was similar at the time they isolated PA to their first sample: 2.3 (1.8-3.0) vs 1.9 (1.0-2.7) ppbv, p=0.32. To increase the overall sensitivity for the detection of PA to >80%, the cut-off for the HCN concentration would have to be reduced to around 3ppbv which would hugely reduce the specificity.

It is unsurprising that the incidence of new PA isolates was highest in the FF-Tx group (0.41 cases per patient year) as although they had not isolated PA for at least 12 months, they had previously been diagnosed with chronic PA infection.

The difference between the Never and FF groups is more interesting. Having not grown PA for >12 month it would be assumed that the previous isolates of PA in the FF group had been successfully eradicated. Despite this the FF group had a slightly higher PA incidence than the Never group. This suggests that either a proportion of the previous PA infections had not been successfully eradicated or that the children were at increased risk due to re-infection possibly from the same source as the earlier PA isolate. The wide variation in PA incidence between the centres (0.08 to 0.28 cases per patient year) has occurred despite all centres having strict infection control measures in place. It is a reminder that constant vigilance is required by patients with CF to reduce their risk of PA infection both inside and outside the hospital. The need for regular microbiology surveillance for children with CF is highlighted by the finding that 42% of children were asymptomatic at the time of the PA isolate.

In summary the SPACE Study has demonstrated that an elevated HCN concentration in exhaled breath is a specific marker of PA infection in children with CF. The sensitivity was less impressive and further work is required to investigate the reasons for this and see if it can be improved.

5.2.4 Funding

We gratefully acknowledge the funding for The SPACE Study from a Research for Patient Benefit Grant from the National Institute of Health Research (PB-PG-0909-20070).

Chapter Six

Hydrogen cyanide as an *in-vivo* marker of *Pseudomonas aeruginosa* in adults

CHAPTER SIX – HYDROGEN CYANIDE AS AN *IN-VIVO* MARKER OF *PSEUDOMONAS AERUGINOSA* IN ADULTS

6.1 STUDY FIVE: HYDROGEN CYANIDE CONCENTRATIONS IN THE BREATH OF ADULT CYSTIC FIBROSIS PATIENTS WITH AND WITHOUT PSEUDOMONAS AERUGINOSA INFECTION

6.1.1 Introduction

To our knowledge, no previous study has investigated the exhaled breath HCN concentration in adults with CF and chronic PA infection. The interpretation of exhaled breath HCN concentrations in adults is more complex than in children, as most healthy adults have easily measurable concentrations of HCN in mouth-exhaled breath.[151,156] This is different to healthy children in whom mouth-exhaled breath HCN concentrations are either very low or immeasurable.[137] Simultaneous studies of both mouth-exhaled and nose-exhaled breath HCN concentrations in healthy adults showed that even when mouth-exhaled HCN concentrations are elevated, nose-exhaled HCN concentrations remained very low or undetectable. These studies proved the site of HCN production in such individuals was the oral cavity.[151,156] Therefore, the investigation of HCN as a breath biomarker for PA infection in adults requires both nose-exhaled and mouth-exhaled HCN concentrations to be measured.

6.1.2 Methods

6.1.2.1 Experimental design

Two groups of non-smoking adult patients with CF were recruited from the Manchester Adult CF Centre; 10 with chronic PA infection who were consecutive patients at the “PA out-patient clinic” and 10 who were free from PA infection that were consecutive patients attending the “non-PA out-patient” clinic. Chronic PA infection was defined as >50% of sputum cultures positive for PA in the previous 12 months (minimum of 4 samples). Free from PA infection was classified as no PA positive sputum cultures in the previous 12 months (minimum of 4 samples). Patients were excluded if they were too short of breath to provide effective breath samples or if they had isolated BCC or a *Pseudomonas* species other than PA in the previous 12 months.

Following written informed consent, patients provided on-line mouth and nose-exhaled breath samples using the methodology described in 3.3.3. Patients were requested not to eat or drink anything for 1 hour prior to breath samples being provided. In addition to HCN, we chose to simultaneously measure acetone and ethanol in the same breath exhalations. Acetone is a systemic compound which means the measured mouth-exhaled and nose-exhaled concentrations are invariably the same when the sampling rate is consistent.[151] This is a valuable indicator of nose and mouth sampling consistency. In contrast, ethanol is partially generated in the mouth by bacterial and salivary enzyme activity resulting in nose-exhaled concentrations being lower than mouth-exhaled concentrations.[151]

6.1.2.2 Statistical methods

SPSS Statistics Version 21 (IBM Corp. Released 2012) was used for the analysis. The concentrations of the acetone, ethanol, HCN and water vapour were not normally distributed, even after logarithmic transformation. Therefore, the results are displayed as median (IQR) values. The Mann-Whitney U test was used to assess the significance of the differences of concentrations between groups. A p-value of <0.05 was deemed significant.

6.1.2.3 Ethical approval

Ethical approval was granted by NRES Committee North West – Haydock Park (11/NW/0102). See Annex 2: 11.2.3. This ethical approval was used for studies 5 and 6.

6.1.3 Results

6.1.3.1 Patient demographics

Ten patients attending the out-patient clinic were recruited into the “chronic PA” group and 10 into the “free-from-PA” group. There were no significant differences in the forced vital capacity (FVC) and body mass index (BMI) between the 2 groups. There were non-significant trends for those with chronic PA infection to be younger and have lower forced expiratory volume in 1 second (FEV1). As expected, the time since the last PA isolate was significantly shorter in the “chronic PA group” compared to the “PA-free” group (0.2 (0.1-0.2) years vs 3.3 (2.5-4.9) years, $p<0.001$). See Table 16.

Table 16: Demographic data on the patients in the *Pseudomonas aeruginosa* (PA) and PA free groups

	Age (years)	**Lung Function**		**BMI**	**Time since last PA isolate (years)**
		FVC (%)	**FEV1 (%)**		
Chronic PA (n=10)	25.8 (21.5-31.5)	50 (38-60)	56 (48-71)	19.9 (18.4-20.8)	0.2 (0.1-0.2)
PA-free (n=10)	31 (24.8-38.5)	56 (48-71)	53 (48-68)	20.5 (19.1-21.8)	3.3 (2.5-4.9)
p value	0.26	0.34	0.14	0.55	<0.001

FVC: forced vital capacity expressed as percentage predicted. FEV1: forced expiratory volume in 1 second expressed as percentage predicted. BMI: body mass index. PA: *Pseudomonas aeruginosa.* p values calculated using the Mann-Whitney U test. All, values median (IQR)

6.1.3.2 Breath analysis results

The concentrations of acetone, ethanol and HCN from mouth-exhaled and nose-exhaled breath are shown for the chronic PA infection group in Table 17 and for the free from PA infection group in Table 18.

Table 17: Breath results from the chronic *Pseudomonas aeruginosa* group

Pt No.	Mouth-exhaled Breath			Nose-exhaled Breath		
	Acetone	Ethanol	HCN	Acetone	Ethanol	HCN
1	605	322	32	592	99	18
2	333	256	33	293	129	16
3	576	188	4.5	583	226	0
4	394	298	17	307	95	11
5	551	257	22	512	167	18
6	557	335	22	626	120	0
7	514	156	31	453	71	20
8	353	839	2.2	363	388	3
9	1033	268	5.7	971	160	0
10	236	930	13	227	675	11
Median	**533**	**283**	**20**	**483**	**145**	**11**

The concentrations for each individual are the means of 3 consecutive breath samples. The median of the 10 individual values are also given for each compound. All concentrations are in parts-per-billion by volume, ppbv.

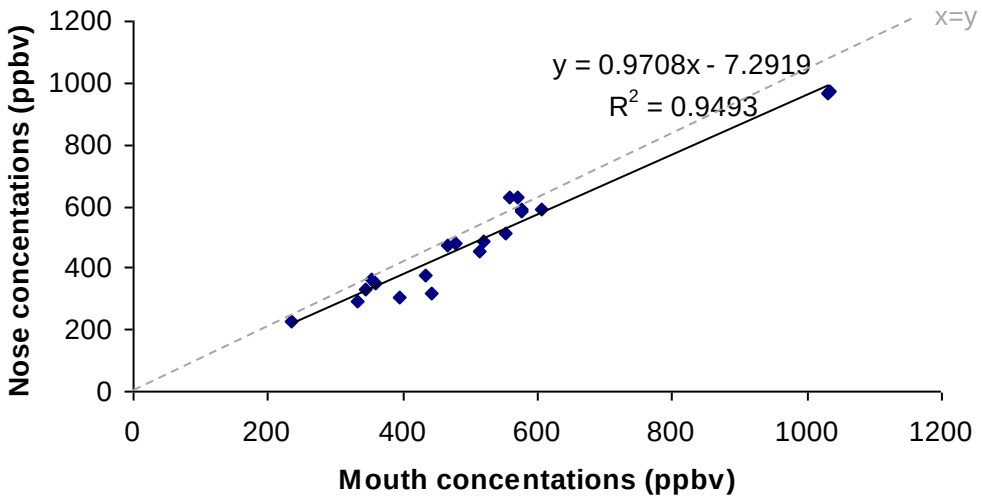
Table 18: Breath results from subjects in the control group

No	Mouth-exhaled Breath			Nose-exhaled Breath		
	Acetone	Ethanol	HCN	Acetone	Ethanol	HCN
1	467	1041	0	471	351	0
2	571	340	23	627	115	0
3	358	3772	2.9	348	426	4.3
4	435	244	17	379	143	0
5	1032	372	0	969	133	0
6	345	388	2.2	330	157	0
7	520	323	39	486	131	11
8	477	998	3.7	481	367	0
9	577	318	21	588	118	0
10	442	321	11.2	321	93	11
Median	**472**	**356**	**7.5**	**476**	**138**	**0.03**

The concentrations for each individual are the means of 3 consecutive breath samples. The median of the 10 individual values are also given for each compound. All concentrations are in parts-per-billion by volume, ppbv.

When the data from all 20 patients (chronic PA and free from PA) were analysed together, the median (IQR) acetone concentrations were similar for mouth and nose-exhaled samples: 496 (385-572) vs 476 (344-589) ppbv, p=0.73). This similarity was confirmed by the excellent correlation (R^2=0.95) when mouth and nose-exhaled acetone concentrations were plotted against each other; see Figure 15. In contrast, but as expected, the median (IQR) ethanol concentrations were higher for mouth-exhaled samples than for nose-exhaled samples: 323 (265-501) vs 138 (117-257) ppbv, p=0.001). A similar trend was seen for the median (IQR) HCN concentrations: 15 (3.5-22) vs 1.5 (0-11) ppbv, p=0.006).

Figure 15: Correlation of mouth-exhaled and nose-exhaled acetone concentations for all patients.



The concentrations of the 3 volatile compounds for both groups are summarised in Table 19. The median (IQR) nose-exhaled HCN concentration was significantly higher in patients with chronic PA infection compared to those free from PA infection: 11 (0.8-18) vs 0 (0-3.2) ppbv, p=0.03. Hypothesis 8 is therefore accepted. There was a non-significant trend for mouth-exhaled HCN to be higher in the chronic PA group: 20 (7.5-29) vs 7.5 (2.3-20) ppbv, p=0.17.

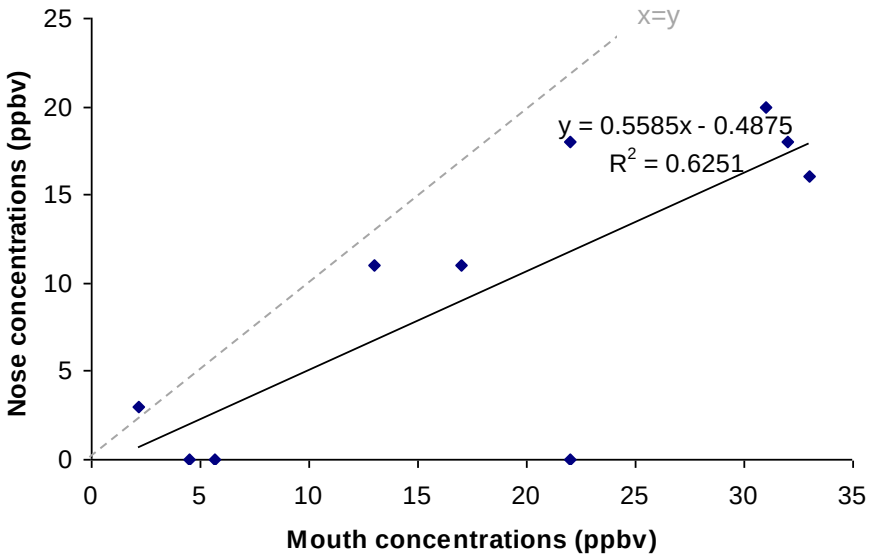
Table 19: Comparison of breath results chronic *Pseudomonas aeruginosa* (PA) and free from PA groups.

	Mouth-exhaled Breath			**Nose-exhaled Breath**		
	Acetone	**Ethanol**	**HCN**	**Acetone**	**Ethanol**	**HCN**
Chronic PA	533 (363-571)	283 (256-332)	20 (7.5-29)	483 (321-590)	145 (104-211)	11 (0.8-18)
Free from PA	472 (437-558)	356 (322-846)	7.5 (2.3-20)	476 (356-563)	138 (121-303)	0 (0-3.2)
P value	1.0	0.06	0.17	0.82	0.88	0.03

Concentration values are median (IQR) ppbv. PA: patients with Pseudomonas aeruginosa infection, No PA: patients without Pseudomonas aeruginosa and Burkholderia Cepacia Complex infection.

When the nose-exhaled and mouth-exhaled HCN concentrations were plotted against each other for patients with chronic PA infection, there was a moderate correlation (R^2=0.63); see Figure 16. Despite the difference in the nose-exhaled HCN concentrations between the 2 groups there were 4 patients with chronic PA infection whose nose-exhaled HCN concentrations were not elevated (all <5ppbv) and 2 patients who were free from PA infection who had elevated nose-exhaled HCN concentrations (>10ppbv). Of the 2 patients with elevated nose-exhaled HCN despite being free from PA, one had not grown PA since attending our unit (7 years previously) and one had grown PA 3.2 years ago that was successfully eradicated.

Figure 16: Correlation of mouth-exhaled and nose-exhaled hydrogen cyanide concentations for patients with chronic *Pseudomonas aeruginosa* infection.



A non-significant trend was also seen for mouth-exhaled ethanol concentrations to be lower in patients with chronic PA compared to those free from PA infection. There were no significant differences between the 2 groups for mouth-exhaled

acetone concentrations, nose-exhaled acetone concentrations or nose-exhaled ethanol concentrations.

6.1.4 Discussion

To our knowledge, this is the first study involving the analysis of the HCN concentration of mouth-exhaled and nose-exhaled breath in adults with CF, with and without PA infection. The nose-exhaled HCN concentrations are significantly higher in those patients with chronic PA infection compared to those free from PA infection and there is a trend for the mouth-exhaled HCN concentration to be higher in those with chronic PA infection. Despite this, there were 4 patients with chronic PA infection whose nose-exhaled HCN concentrations were not elevated (all <5ppbv) and 2 patients who were free from PA infection who had elevated nose-exhaled HCN concentrations (>10ppbv). Although we recognise the small patient cohorts involved, this suggests that for the detection of PA infection in adults with CF, elevated nose-exhaled HCN concentration has a sensitivity of 60% and a specificity of 80%.

With regards to the sensitivity; it is known that there is variability in the HCN production by different strains of PA cultured *in-vitro.*[187] Although this has not been directly correlated with *in-vivo* studies, it is likely that infection with a low HCN producing strain will result in lower breath HCN concentrations. It is also known there is a diurnal variation in HCN production[196] and when multiple breath samples are taken from CF patients with chronic PA infection, there is a significant intra-subject variability in HCN concentrations.[136] As patients only provided breath samples at a single visit, it is possible that we limited the opportunity to detect an elevated HCN concentration. The anticipated increase in sensitivity of forthcoming

SIFT-MS instruments will improve the sensitivity of this diagnostic test. With regard to the specificity, it is known that patients can have chronic PA infection that is not detected by routine cough swabs or sputum culture and are only diagnosed by the more invasive sampling of bronchoalveolar lavage.[107] It is therefore possible that those patients in the PA-free group who had elevated nose-exhaled HCN concentrations were actually infected with PA.

The median mouth-exhaled HCN concentrations of 7.5ppbv seen in adults free from PA infection is close to a previous report of 10 ppbv in a cohort of healthy volunteers.[156] The median (IQR) concentrations in mouth-exhaled breath for CF adults with chronic PA infection was 20 (7.5-29) ppbv. This is higher than that in the mouth-exhaled breath of children with CF and PA infection, which we previously reported as 13.5 (8.1-16.5) ppbv.[136] The reason for this difference is not clear, but it may reflect some additional oral production of HCN in the adults with CF, as we have also shown in healthy adults. It is also necessary to consider the possible effect of sinus infection on breath analysis results. It is now increasingly recognised that patients with CF can have chronic PA infection in their sinuses.[197] In this study we did not take sinus samples to check for this type of infection. If PA sinus infection was present in the absence of pulmonary infection then this may be the cause of a presumed false positive. It is also possible that coexisting sinus and pulmonary infection may result in a different pattern of volatiles such as a higher HCN. Further work is required to look into this interesting area.

The acetone concentrations were remarkably similar for mouth-exhaled and nose-exhaled breath and when plotted against each other, the correlation is excellent (R^2 = 0.95); see Figure 15. The slope of the linear plot was 0.97, indicating

marginally lower concentrations in the nose-exhaled breath. The median mouth-exhaled acetone concentration across both groups was 496 ppbv which is close to the 477 ppbv reported in a 6-month longitudinal study involving 30 healthy adults.[158] These results evidence the consistency of the mouth and nose sampling, but also the remarkable consistency of the results obtained for breath acetone concentrations obtained for two different adult cohorts using two different SIFT-MS instruments. Whilst the factors that can influence breath acetone concentrations are not the focus of this thesis, they are well understood.[23,24]

Whilst there is wide variation between the ethanol concentrations measured in mouth-exhaled breath, nose-exhaled ethanol concentration are lower than the mouth-exhaled concentrations in all but one patient. This difference relates to the oral production of ethanol. The median mouth-exhaled ethanol concentrations in patients free from PA infection are skewed higher due to the excessive concentrations seen in 3 patients (see Table 18). Sugary drinks can elevate mouth-exhaled ethanol as does the recent ingestion of alcohol.[159,199] Although patients were requested not to eat or drink for one hour prior to providing breath samples, this was not monitored. The ethanol concentrations found in this study are similar to those previous determined in the exhaled-breath of healthy cohorts.[159] It is noteworthy that both ethanol and acetone concentrations in exhaled breath condensate (analysed using nuclear magnetic resonance spectroscopy) are different between patients with CF and healthy subjects.[200]

In summary, this study shows that nose-exhaled HCN concentrations are significantly higher in adult patients with CF who have chronic PA infection than in adult patients with CF free from PA infection. HCN is present in the mouth-

exhaled breath of both groups and a proportion of this is likely to have been generated in the oral cavity. When measuring HCN as a marker of PA infection in the lower airways of adults, nose-exhaled breath should be used since this bypasses the oral cavity. The use of mouth-exhaled HCN is appropriate in children, as they do not generate measurable HCN in the oral cavity.[137] Improvements in oral hygiene in adults can reduce the concentrations of some orally generated compounds.[199,201] It may be possible in the future to use mouth-exhaled HCN concentrations in adults if their oral hygiene is maximised. Our results support the use of HCN as a breath biomarker of PA infection, but more studies involving larger patient cohorts are needed to assess its full potential as a non-invasive diagnostic test for early infection.

Chapter Seven

Hydrogen cyanide as a marker of *Burkholderia cepacia complex*

CHAPTER SEVEN – HYDROGEN CYANIDE AS A MARKER OF *BURKHOLDERIA CEPACIA COMPLEX*

7.1 STUDY SIX: IS HYDROGEN CYANIDE AN *IN-VITRO* OR *IN-VIVO* MARKER OF *BURKHOLDREIA CEPACIA COMPLEX* INFECTION?

7.1.1 Introduction

It was thought that PA was the only organism found in the CF lung to produce HCN, making it a possible specific marker of PA infection. A recent paper used a cyanide ion-selective micro-electrode to demonstrate the production of the non-volatile cyanide ion by *Burkholderia cepacia complex* (BCC) when cultured under biofilm but not planktonic conditions.[130] BCC refers to a group of at least 17 closely related bacterial species (formerly called genomovars) that can cause pulmonary infection in patients with CF.[202,203] We replicated the methodology of *in-vitro* experiment but rather than using a cyanide electrode to measure cyanide trapped in sodium hydroxide, we used selected ion flow tube mass spectrometry (SIFT-MS) to measure the headspace concentration of HCN. In addition we investigated if HCN is an *in-vivo* marker of BCC infection by measuring its concentration in mouth and nose-exhaled breath in adults with CF and chronic BCC infection.

7.1.2 Methods

7.1.2.1 Experimental design

Adult patients with CF were recruited if they had chronic BCC infection and were free from PA infection. Chronic BCC infection was defined as BCC in >50% of sputum samples (minimum of 4 samples) over the previous 12 months, with a.

Free from PA infection was defined as no PA isolate in the previous 12 months (minimum of 4 samples). Once recruited, patients provided on-line mouth and nose-exhaled breath samples for HCN analysis using the methodology described in 3.3.3. They also provided a sputum sample. The sputum sample was cultured using the methodology described in 3.2.2.1. Each BCC sample was then used to prepare a biofilm and planktonic culture using the methodologies described in 3.2.2.2 and 3.2.2.3 respectively. Biofilm and planktonic control cultures were prepared using the methodology described 3.2.3. Biofilm formation was confirmed visually and using spectrophotometry as described in 3.2.4.

The headspace HCN concentration of the BCC biofilm, planktonic and control cultures were measured at 24, 48, 72 and 96 hours of incubation using the methodology described in 3.3.6. At 96 hours the headspace HCN concentrations were re-measured after acidification of the cultures using 1ml HCl to promote the generation of HCN from cyanide ions. The mouth and nose-exhaled breath of a group of patients free from BCC and PA infection were analysed as controls.

7.1.2.2 Ethical approval

Ethical approval was granted by NRES Committee North West – Haydock Park (11/NW/0102). See Annex 2: 11.2.3. This ethical approval was used for studies 5 and 6.

7.1.3 Results

7.1.3.1 Patient demographics

Twelve patients (6 male) were recruited from the Manchester Adult CF Centre that had chronic BCC infection and were free from PA infection (BCC Group) and 10

patients (6 male) were recruited that were free from both BCC and PA infection (control group). There were no significant differences between the groups in age, body mass index (BMI) and forced vital capacity (FVC). The median (IQR) forced expiratory volume in one second (FEV1) was significantly lower patients in the BCC group compared to those in the control group (1.7 (1.4-2.0) vs 2.2 (2.0-2.8) litres, p=0.04). See Table 20. In the BCC group 7/12 had never isolated PA since transferring to the Manchester Adult CF Centre, the median (IQR) time since last isolation of PA in the remaining 5 patients was 2.3 (1.9-2.3) years. Seven were chronically infected with *Burkholderia multivorans*, 3 with *Burkholderia cenocepacia* and 2 with *Burkholderia latens.* These BCC species were confirmed by the National Reference Laboratories within the last 12 months using pulsed field gel electrophoresis, recA sequencing or species specific PCR. In the control group 4 patients had never isolated PA since attending the Manchester Adult CF Centre. In the remaining 6 patients the median (IQR) time since last PA isolation was 3.0 (2.5-3.3) years. None of the control group had isolated BCC since attending the adult unit.

Table 20: Demographics of patients in *Burkholderia cepacia complex* and control groups

Group	Age (years)	Body Mass Index	Lung function	
			FEV1 (L)	FVC (L)
BCC	26.0 (22.8-36.1)	22.2 (19.8-23.0)	1.7 (1.4-2.0)	2.9 (2.1-3.1)
Control	31.0 (24.8-38.5)	20.5 (19.1-21.8)	2.2 (2.0-2.8)	2.8 (2.4-3.5)
p value	0.51	0.29	0.04	0.43

BCC: Burkholderia cepacia complex. FEV1: forced expiratory volume in 1 second. FVC: forced vital capacity. All values are median (IQR)

7.1.3.2 *In-vitro* results

Biofilms were identified visually on all biofilm cultures after 48 to 96 hours incubation. The mean (SD) absorbance of crystal violet was measured after 96 hours incubation using spectrophotometry. This was higher in the biofilm cultures compared to the planktonic and control cultures (3.43 (0.31) vs 0.005 (0.003) Absorbance Units (AU), $p<0.001$), confirming biofilm formation. The headspace HCN concentration measured using SIFT-MS remained <10ppbv (equivalent to background concentrations) for both culture conditions, at all time points, see Table 21. Hypothesis 9 is therefore rejected. After acidification there was a rise in the median (IQR) headspace HCN concentration compared to the pre-acidification 96 hour results (4.5 (3.8-5.5) vs 2.85 (1.5-3.5) ppbv, $p=0.002$) but all concentrations remained <10ppbv. Acidification did not produce a significant change in the median (IQR) headspace HCN concentrations for the planktonic cultures (2.3 (1.7-3.4) vs 1.7 (1.4-3.4) ppbv, $p=0.6$).

Table 21: Headspace hydrogen cyanide concentrations for biofilm and planktonic cultures after various durations of incubation

Pt no.	Species	Culture conditions	HCN (ppbv)					Mean (SD) absorbance of crystal violet (AU)
			24 hrs	48 hrs	72hrs	96 hrs	96 hrs & HCl	
1	*Burkholderia latens*	BF	5.2	4	2	5.0	7.0	3.55 (0.45)
		Pl	3.3	1.6	3.9	1.5	1.9	0.01 (0.01)
2	*Burkholderia multivorans*	BF	0.2	0.5	2.2	3.6	6.3	3.68 (0.42)
		Pl	3.2	2.2	3.6	5.2	3.6	0.003 (0.001)
3	*Burkholderia multivorans*	BF	0.2	1.7	2.0	3.4	3.9.	3.52 (0.48)
		Pl	1.4	0.2	3.3	1.6	1.3	0.01 (0.01)
4	*Burkholderia cenocepacia*	BF	3.2	1.8	1.2	3.0	4.9	3.48 (0.72)
		Pl	0.2	1.7	3.0	0.2	3.3	0.004 (0.003)
5	*Burkholderia cenocepacia*	BF	3.4	4.7	1.9	1.4	3.3	3.83 (0.29)
		Pl	2.5	3.8	2.8	2.7	3.3	0.003 (0.001)
6	*Burkholderia latens*	BF	0.1	2.8	1.1	1.5	4.5	3.75 (0.33)
		Pl	1.2	3.9	4.9	1.4	1.8	0.003 (0.001)
7	*Burkholderia multivorans*	BF	0.1	1.5	2.2	0.1	4.4	3.77 (0.25)
		Pl	1.6	0.2	3.3	1.7	2.0	0.003 (0.002)
8	*Burkholderia cenocepacia*	BF	0.5	1.1	2.5	1.6	3.4	3.33 (0.66)
		Pl	2.3	3.6	5.2	1.4	1.1	0.003 (0.001)
9	*Burkholderia multivorans*	BF	0.5	0.4	4.9	3.7	7.8	3.12 (0.81)
		Pl	0.9	2.5	3.1	1.4	1.3	0.003 (0.001)
10	*Burkholderia multivorans*	BF	2.2	2.5	3.1	2.8	5.2	3.09 (0.70)
		Pl	0.8	2.7	3.9	4.2	5.7	0.004 (0.001)
11	*Burkholderia multivorans*	BF	0.2	2.7	1.8	1.2	4.2	3.17 (0.70)
		Pl	1.5	2.1	3.1	4.5	2.6	0.003 (0.001)
12	*Burkholderia multivorans*	BF	2.1	2.5	2.1	2.9	2.2	2.89 (0.60)
		Pl	5.1	2.4	3.8	4.2	4.4	0.002 (0.001)
	Sterile Control	BF	0.1	0.5	1.2	0.1		0.06 (0.04)
		Pl	0.3	1.2	2.2	3.4		0.05 (0.04)
	Sterile Control	BF	0.3	3.6	3.1	0.9		0.06 (0.06)
		Pl	0.2	0.8	2.2	3.1		0.06 (0.05)

Pt no: patient number, BF: biofilm, Pl: planktonic, ppbv: parts per billion by volume, HCN: hydrogen cyanide, HCl: hydrochloric acid, SD: standard deviation, AU: absorbance units

7.1.3.3 *In-vivo* results

When data from all 22 patients (with and without BCC) was analysed together, the systemic compound acetone had similar median (IQR) concentrations for mouth and nose-exhaled samples (459 (401-557) v 445 (354-567) ppbv, p=0.60). In contrast, the median (IQR) concentrations of ethanol and HCN, which are known to be generated in the mouth, were higher in mouth-exhaled breath samples than nose-exhaled breath samples (HCN: 6.8 (1.2-20) v 0 (0-0.9) ppbv, p<0.001 and ethanol: 410 (346-555) v 152 (129-318) ppbv, p<0.001). See Tables 22 and 23.

When the acetone, ethanol and HCN concentrations were compared between the BCC and control groups, there were no significant differences for nose-exhaled or mouth-exhaled samples. See Table 24. The absence of a difference in the nose-exhaled HCN concentrations between the BCC and control groups leads to Hypothesis 10 being rejected.

Table 22: *In-vivo* results for subjects in the *Burkholderia cepacia complex* group

No	Mouth Exhaled Breath			Nose Exhaled Breath		
	Acetone	**Ethanol**	**HCN**	**Acetone**	**Ethanol**	**HCN**
1	363	2730	0	350	429	0
2	450	278	17	410	153	0
3	569	248	9.6	597	151	0
4	1062	250	1.0	965	128	0
5	441	362	4.0	330	157	0
6	512	316	37	503	134	2.2
7	569	341	23	615	113	0
8	429	856	29	419	767	0
9	481	1103	1.8	479	370	0
10	298	289	10	321	123	3.3
11	391	488	0	366	219	1.2
12	375	272	0	371	187	0

Concentration values are parts-per-billion by volume

Table 23: *In-vivo* results for subjects in the control group

No	Mouth Exhaled Breath			Nose Exhaled Breath		
	Acetone	**Ethanol**	**HCN**	**Acetone**	**Ethanol**	**HCN**
1	467	1041	0	471	351	0
2	571	340	23	627	115	0
3	358	3772	2.9	348	426	4.3
4	435	244	17	379	143	0
5	1032	372	0	969	133	0
6	345	388	2.2	330	157	0
7	520	323	36	486	131	11
8	477	998	3.7	481	367	0
9	577	318	21	588	118	0
10	442	321	11.2	321	93	6

Concentration values are parts-per-billion by volume

Table 24: Comparison of *in-vivo* results between the *Burkholderia cepacia complex* and control groups

	Mouth-exhaled Breath			Nose-exhaled Breath		
	Acetone	**Ethanol**	**HCN**	**Acetone**	**Ethanol**	**HCN**
BCC	446 (387-526)	446 (387-526)	6.8 (0.8-19)	415 (362-527)	155 (133-257)	0 (0-0.3)
Controls	472 (437-558)	356 (322-846)	7.5 (2.3-20)	476 (356-563)	138 (121-303)	0 (0-3.2)
p value	0.55	0.26	0.74	0.79	0.37	0.53

BCC: *Burkholderia cepacia complex*. Concentration values are median (IQR) parts-per-billion by volume.

7.1.4 Discussion

Using SIFT-MS, we did not identify elevated HCN concentrations in the headspace of biofilm or planktonic BCC cultures at any time point. This included analysis at a time when biofilm formation had been confirmed visually and with spectrophotometry. We are unable to explain why our results are inconsistent with the liquid-phase cyanide ion production in biofilm cultures demonstrated by Ryall *et al.*[130] but conclude that HCN is not a reliable *in-vitro* marker of BCC infection. To our knowledge, the current study is the first to investigate gaseous HCN as an

in-vivo marker of BCC infection. Since both mouth and nose-exhaled HCN concentrations were no higher in those with chronic BCC infection than in controls, this again suggests that HCN is not an *in-vivo* marker of BCC infection.

In the paper by Ryall *et al.* 32 of 34 BCC samples were found to be cyanogenic with the concentrations of trapped cyanide ranging from 60µM to 19mM (equivalent to equilibrium headspace HCN gas concentrations of 7 ppmv to 2100 ppmv at 20 C) [204]. If our cultures of BCC produced similar concentrations of HCN, they would be easily detected using SIFT-MS. Acidification by HCl of the BCC cultures produced a significant increase in the gaseous HCN concentrations suggesting that some cyanide ions were present which, on acidification, are converted to HCN that is partially partitioned into the gas phase. Despite this, the HCN concentrations remained <10ppbv, meaning that the scale of the HCN production was not close to that of the cyanide production seen by Ryall *et al.* The reason for this difference is not clear. Ryall *et al.* used 34 BCC samples (from 9 species) which were a mixture of clinical and environmental isolates from the BCCM/LMG collection in Ghent. They found a greater than 2 log variation in the cyanide concentrations for the different BCC species and a greater than 5 log variation when the concentrations were normalised to the colony forming unit counts. We assessed 12 BCC samples from 3 species, all were clinical isolates. The different origin of the samples and the different species analysed may therefore be contributing factors. Methodological issues also need to be considered, but both the cyanide ion-selective micro-electrode used by Ryall *et al*. and our SIFT-MS instrument have been previously been successfully used when investigating the cyanide / HCN production by PA. It is also possible that the BCC cultures are producing a compound that chelates the HCN of that the HCN is

remaining trapped in the biofilm. Both of these possibilities would prevent HCN from being released into the gas phase and therefore the headspace HCN concentration would not become elevated.

Although further work needs to be undertaken to investigate the apparent discrepancy between the *in-vitro* production of cyanide ions and HCN by BCC cultures, our ultimate aim is to identify a biomarker that could be used in a breath test to diagnose BCC infection. This is clinically important, as patients with CF who acquire BCC are known to have an accelerated loss of lung function, more hospital admissions and increased mortality.[205] Despite this, there is a wide spectrum of clinical manifestations and it can be difficult to diagnose especially in patients who do not expectorate sputum.[206,207] As cyanide ions are not volatile, they cannot be measured in exhaled breath, so any breath test based on the cyanogenic properties of BCC would therefore have to use gaseous HCN as its marker. As we did not find any difference in the nose- or mouth-exhaled HCN concentrations between the BCC and control groups, there is no indication that HCN is an *in-vivo* marker of BCC infection.

In summary we did not identify elevated HCN concentrations in the headspace of BCC samples cultured under planktonic or biofilm condition or in the breath of patients chronically infected with BCC. We therefore conclude that HCN is not an *in-vitro* or *in-vivo* marker of BCC infection. Further work needs to be undertaken to investigate the apparent discrepancy between the *in-vitro* production of cyanide ions and HCN by BCC.

Chapter Eight

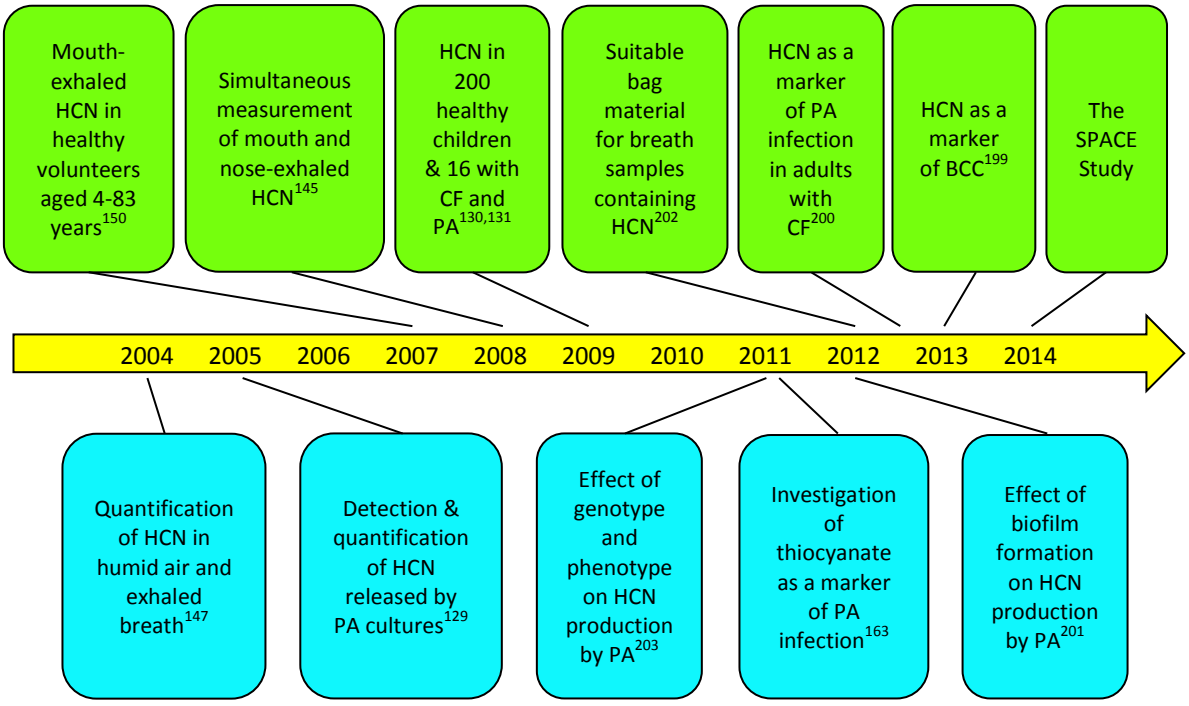
Conclusions

CHAPTER EIGHT – CONCLUSIONS

8.1 THE CONTEXT OF THIS THESIS

As can be seen from the timeline in Figure 16, the work included in this thesis is the culmination of a decade of research using SIFT-MS to investigate HCN as a marker of PA infection. This work has been devised and supervised by Professors Warren Lenney, David Smith and Patrik Španěl and undertaken at the University Hospital of North Staffordshire / Keele University and the J. Heyrovský Institute of Physical Chemistry, Prague. Significant contributions to this work have also been made by Dr Will Carrol, Dr Beth Enderby, Violetta Shestivska and Ksenia Dryahina.

Figure 17: Timeline of work using SIFT-MS to investigate hydrogen cyanide as a marker of *Pseudomonas aeruginosa* infection (plus related studies)



In-vivo studies are in green and *in-vitro* in blue. HCN: hydrogen cyanide, CF: cystic fibrosis, PA: *Pseudomonas aeruginosa,* BCC: *Burkholderia cepacia complex.*

8.2 HYDROGEN CYANIDE AS AN *IN-VITRO* MARKER OF *PSEUDOMONAS AERUGINOSA* INFECTION

The overall aim of the *in-vitro* studies in this project was to identify factors which affected HCN production. We hoped that this would provide more data to inform the possible development of an *in-vivo* breath test for the detection of PA. It was already known that cyanide synthesis by PA occurs under microaerobic (O2 <5%) conditions[131] and that it was maximised at temperatures between 34^{o}C and 37^{o}C.[132] The effect of genotype had not previously been investigated although a possible effect was suggested as the cyanide concentrations produced by cultures of PA were independent of bacterial load.[133] Previous studies had reported some data on the effect of phenotype of cyanide production suggesting higher production by mucoid PA cultures.[150,187]

Study 1 clearly shows that different strains of PA produce different quantities of HCN. It also demonstrates that phenotype has an effect but in contrast to the studies that measure cyanide production, non-mucoid isolates produced more HCN than mucoid isolates. There are a number of possible explanations for these findings. The effect of phenotype varied according to strain and the timing of analysis also had an effect. One hypothesis that has not previously been considered is that the mucoid PA cultures produce more cyanide (as found in the previous studies) but the layer of alginate prevents the release of HCN into the gas phase. These factors may therefore have different effects on the production of cyanide and the production of HCN. As any future *in-vivo* breath test for PA detection is likely to be based on the analysis of a volatile compound, we are especially interested in the factors that affect HCN (volatile) rather than cyanide (non-volatile).

The effect of biofilm formation was investigated with PA cultures in Study 2 and BCC cultures in Study 6. As HCN is a quorum sensing molecule we expected the formation of biofilms to increase the production of HCN by PA and turn on the production of HCN by BCC (as seen with cyanide[130]). Despite this, in Study 2 we found no significant increase in the amount of HCN produced by biofilm PA cultures compared to planktonic PA cultures and in Study 6 no HCN was produced by either the planktonic or biofilm BCC cultures. One possible explanation for this is related to the hypothesis regarding the production of HCN by non-mucoid and mucoid PA cultures. This is that the PA biofilm cultures are producing cyanide but this remains trapped within the biofilm and is therefore not released as HCN into the gas phase and is therefore not detected in the culture headspace. If this were the only explanation then a bigger increase in HCN concentration may have been expected when the BCC cultures in study 6 were acidified. Further work needs to be done to investigate the effect of biofilm formation on HCN production.

One of the major difficulties when measuring the volatiles in bacterial culture headspace is assessing the effect of the initial bacterial mass and possible different growth rates. Our techniques have evolved to try and account for these factors. In Study 1 we had no way of attempting to correct for this as we used plate cultures. In Study 2 the preparation of liquid cultures will have produced a more standardised initial bacterial mass and in Study 6 the liquid culture was serially diluted to achieve a turbidity of 0.5 optical density units measured by spectrophotometry which correlates to 10^8 CFU/ml. Although this standardises the initial bacterial mass, varying growth rates means that the bacterial mass may vary at the time that the cultures are analysed. More recent studies (not included in this thesis) have tried to measure bacterial mass at the time of headspace analysis.[208]

In general, the results of the *in-vitro* studies have supported the development of an *in-vivo* HCN breath test for the early detection of PA. Early PA infection tends to occur with an organism that has a non-mucoid phenotype and grows under planktonic conditions. Our studies have shown that HCN production is higher amongst non-mucoid compared to mucoid cultures and is no lower in planktonic compared to biofilm cultures. The variation in HCN production according to PA genotype means that some strains produce less HCN. It is possible that infection with one of these strains would be less likely to be identified by an *in-vivo* HCN breath test for PA detection. As yet, the exact relationship between the *in-vitro* and *in-vivo* HCN concentration has not been determined. The failure of BCC to produce HCN means that PA is the only organism frequently found in the CF lung that produces HCN.

8.3 HYDROGEN CYANIDE AS AN *IN-VIVO* MARKER OF *PSEUDOMONAS AERUGINOSA* INFECTION

The culmination of my PhD project was The SPACE Study (Study 4) which investigated if HCN was an early marker of PA infection in children with CF. I am very proud of the high standards that were achieved for this large, multi-centre observational study and of the large quantity of useful data that it generated. The specificity of breath HCN concentration as a marker of PA infection was reassuring although the relatively low sensitivity was ultimately disappointing. In its current form the low sensitivity precludes it from being used as a screening test for PA infection although the high specificity suggests that it may still have a role in the confirmation of PA infection. Further work needs to be undertaken to fully understand the factors that limited the sensitivity. The genotype of the infecting PA is one important factor and specifically it would be useful to know if the children

with a false negative HCN result were infected with a strain of PA known to produce less HCN when cultured *in-vitro.* Unfortunately, we did not have adequate funding to undertake genotyping or *in-vitro* analysis of HCN production on the new PA isolates as part of The SPACE Study. There are methodological issues that may have contributed to the low sensitivity. It would be useful to know if the rate of false positive would have been lower if we had used on-line breath analysis. Although this is not possible with one SIFT-MS instrument in a multi-centre study, there are a number of commercial companies that have stated an interest in developing a portable breath HCN monitor.

As a percentage of healthy adults produce HCN in their oral cavity, it was previously thought that breath HCN could not be used as a marker of PA infection in adults with CF. Study 5 and 6 are the first studies in adults with CF to measure the mouth-exhaled and nose-exhaled breath concentrations of a number of volatile compounds. Clear differences can be seen between the systemic compound acetone, which has similar concentrations in mouth and nose-exhaled breath and compounds which to some degree are generated in the mouth (ethanol and HCN) and therefore have high mouth-exhaled concentrations. Study 5 also demonstrated that nose-exhaled HCN was higher in patients with chronic PA compared to those free from PA infection although the sensitivity in this small sample was only 60%.

8.4 IDEAS FOR FUTURE RESEARCH

It would be interesting to know the exact relationship between the headspace HCN concentration measured above *in-vitro* PA cultures and the *in-vivo* exhaled breath HCN concentration of patients infected with the same PA genotype. This could be

investigated relatively easily by the on-line measurement of exhaled breath HCN in a group of patients with chronic PA infection. Sputum samples or cough swabs could be taken from the same patients and cultured. The isolated PA would then be re-cultured and the headspace HCN concentration analysed as previously. This simultaneous *in-vitro* and *in-vivo* analysis was planned with BCC in Study 6 but unfortunately they did not produce any HCN.

The effect of culture phenotype and biofilm formation on cyanide and HCN production needs to be clarified. The production of the non-volatile ion cyanide has been shown to be higher in PA isolates with a mucoid phenotype whereas Studies 1 and 2 demonstrate higher HCN production with non-mucoid compared to mucoid PA cultures. I have hypothesised that PA phenotype may have a different effect on cyanide compared to HCN production. This hypothesis could be tested by simultaneous measurement of cyanide (using a cyanide ion-selective micro-electrode) and headspace HCN (using SIFT-MS) on identical PA cultures. The effect of biofilm formation on cyanide and HCN production by BCC also needs to be clarified. Ryall *et al* demonstrated cyanide production by biofilm but not planktonic BCC[130] whereas in Study 6 no HCN was produced by either the biofilm or planktonic BCC cultures. I have contacted the laboratory that undertook the work on cyanide and BCC and we have discussed the possibility of swapping BCC cultures and undertaking further analysis.

Further work needs to be undertaken to investigate the factors that caused the false negative results in The SPACE Study. The first aspect of this is assessing how the genotype of the infecting PA can affect the *in-vivo* exhaled breath HCN. The second aspect is how the methodology of the exhaled breath HCN can be

refined. In particular it would be interesting to know if on-line SIFT-MS measurement of HCN or its detection using a portable monitor would increase the overall sensitivity.

Chapter Nine

A Personal Footnote

CHAPTER NINE – A PERSONAL FOOTNOTE (OMITTED)

9.1: Delays in Obtaining NHS Permission for Paediatric Clinical Trials (Omitted)

Chapter Ten

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CHAPTER TEN - REFERENCES

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Chapter Eleven

Annexes

CHAPTER ELEVEN – ANNEXES

11.1 ANNEX 1: THE SPACE STUDY CLINICAL DETAILS QUESTIONNAIRE

University Hospital of NHS
North Staffordshire
NHS Trust

Clinical Details Proforma – SPACE study

Centre Number …… Date: ……
Patient Identification Number ……. Visit Number: ……..

1) Since you were last seen in clinic has your child been:
   a. Very well [ ]
   b. Well [ ]
   c. OK [ ]
   d. Not as well as normal [ ]
   e. Unwell [ ]

2) Since you were last seen in clinic has there been any change in the following symptoms:
   a. Cough Increase [ ] No change [ ] Decrease [ ]
   b. Sputum production Increase [ ] No change [ ] Decrease [ ]
   c. Shortness of Breath Increase [ ] No change [ ] Decrease [ ]
   d. Exercise Tolerance Increase [ ] No change [ ] Decrease [ ]

3) Since you were last seen in clinic have you been prescribed any oral antibiotics?
   a. No [ ]
   b. Yes [ ] Name ……
      Dose ……
      Duration ……

4) Since you were last seen in clinic have you been prescribed any nebulised antibiotics?
   a. No [ ]
   b. Yes [ ] Name ……
      Dose ……
      Duration ……

5) Since you were last seen in clinic have you received any intravenous antibiotics?
   a. No [ ]
   b. Yes [ ] Name ……
      Dose ……
      Duration ……

11.2 ANNEX 2 - RESEARCH AND ETHICS APPROVAL

11.2.1 Ethical Approval - Study 3

NHS
Staffordshire Research Ethics Committee
Prospect House
Fishing Line Road
Redditch
Worcestershire
B97 6EW

Tel: 01527 582535
Fax:

16 November 2010

Prof Warren Lenney
Consultant paediatric respiratory medicine
University Hospital North Staffordshire
North Staffordshire Medical Institute,
Hartshill Road,
Stoke- on- Trent,
ST4, 7NY

Dear Prof Lenney

Study title:	**Analysis of Breath in Cystic Fibrosis for Diagnosis of Infection**
REC reference:	**05/Q2604/8**
Amendment number:	**AM03/1 modified**
Amendment date:	**01 November 2010**

Thank you for submitting the above amendment, which was received on 04 November 2010. It is noted that this is a modification of an amendment previously rejected by the Committee (our letter of 26 October 2010 refers).

The modified amendment was reviewed by the Sub-Committee in correspondence. A list of the members who took part in the review is attached.

Ethical opinion

Favourable Opinion

I am pleased to confirm that the Committee has given a favourable ethical opinion of the modified amendment on the basis described in the notice of amendment form and supporting documentation.

The Committee have noted a minor inconsistency in the Funding Organisation between the Young Person's Information sheet and the Parent Information Sheet. The Young Person's sheet states 'Research Grant from the Guy Hilton Research Centre' and the Parent's sheet state 'this hospital's Paediatric Research Fund'. The Committee ask that the inconsistency is rectified.

The Committee also requests that two minor changes are made in the explanatory letter. The second sentence states 'This study is trying to show it is possible...'. The Committee ask that this is changed to '..investigating to see if it is possible..'. In the first line of the second paragraph the word 'the' is missing before 'best'.

The changes requested above are minor amendments and only require that the REC is informed of the changes and copies of revised documents supplied for our files.

The Committee suggests that a letter for the children/young people (written in appropriate language) is produced to address the same issue as the letter approved for the parents. This would require a substantial amendment.

Approved documents

The documents reviewed and approved are:

Document	Version	Date
Letter to participants already enrolled	v1	01 November 2010
Participant Consent Form: Consent Form	v3	02 September 2010
Participant Information Sheet: PIS	v4	02 September 2010
Participant Information Sheet: PIS Children / Young Person	v4	02 September 2010
Protocol	v2	02 September 2010
Modified Amendment	AM03/1 modified	01 November 2010
Covering Letter		01 November 2010

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q2604/8:	**Please quote this number on all correspondence**

Yours sincerely

Mrs Jenny Tyers
Committee Co-ordinator

E-mail: jenny.tyers@westmidlands.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Dr Darren Clement*
Medical Institute
University Hospital North Staffordshire
Hartshill Road
Stoke-on-Trent
ST4 7NY

11.2.2 Ethical Approval - Study 4

National Research Ethics Service
Coventry & Warwickshire Research Ethics Committee
Prospect House
Fishing Line Road
Enfield
Redditch
B97 6EW

Telephone: 01527 582 533
Facsimile: 01527 582 540

07 December 2010

Prof Warren Lenney
Paediatric Respiratory Consultant
University Hospital of North Staffordshire
Academic Department of Child Health
University Hospital of North Staffs
Newcastle Road, Stoke-On-Trent
ST4 6QG

Dear Professor Lenney

Study Title: **The Specificity of Pseudomonas Aeruginosa detection by hydrogen Cyanide concentration in Exhaled breath (The SPACE study)**

REC reference number: **10/H1211/48**

The Research Ethics Committee reviewed the above application at the meeting held on 24 November 2010. Thank you for attending to discuss the study.

Ethical opinion

Discussion

The committee had a number of issues that are detailed in the discussion with you below:

- The Committee asked how children of such a young age would cope with taking part in the breath test when it is like blowing up a balloon? You informed the committee that it is more like a polythene bag so the resistance is very low and we have used it in school with very young children and they coped very well with it.

- The Committee asked, what infection controls are in place for the reusing of the breath testing equipment? You explained that the tube and the filter will be discarded and the bag will be cleaned and tested for infection prior to the next participant using it.

- The Committee wanted to know if the researcher had considered the inclusion of pictures in the 2 to 5 years old information sheet to explain what will happen? You informed the committee that they have the assistance of a co-ordinator who is part of a group that meets with children to get their opinion of the information sheets. You also rely on the parents to discuss the information with their children and explain anything they don't understand.

- The Committee asked if it is totally necessary for the word 'Cyanide' to be included in the title? You informed the committee that this title has been used for another study some years ago and we had no complaints regarding it.

- The Committee asked if a response letter had been sent to the NIHR addressing their concerns? You said that a response letter had been sent to the NIHR and explained in full each point of concern and how these had been addressed.

- The Committee asked if the researcher had considered changing the words 'Mom and Dad' to 'Parent/Guardian' in the information sheets? You said this had not been thought of but would be happy to change this.

- The Committee suggested mentioning that this is a PhD study in the information sheets. The committee also recommended the researcher go to the NRES website to look at the standard format for the information sheets.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

Other Conditions specified by the REC

- The information sheets should be in the standard NRES format available at www.nres.npsa.nhs.uk
- The young children's information sheets should include pictures to explain what will happen
- Change the words 'Mom and Dad' to 'Parent/Guardian' in the children's information sheets?
- In the children's information sheets the words 'Mom and Dad' should be changed to 'Parent/Guardian or Carer'

Should the applicant require further advice the Co-ordinator should be contacted.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Protocol	V1	21 October 2010
Participant Information Sheet: PIS - Young Person Information Sheet	V1	21 October 2010
Letter of invitation to participant	V1	21 October 2010
REC application		25 October 2010
CV for Student - Dr Francis Gilchrist		
Letter from NIHR - Funder		09 August 2010
Participant Information Sheet: PIS - Child Information Sheet	V1	21 October 2010
Referees or other scientific critique report		
Investigator CV		
Participant Information Sheet: PIS - Parent/Carer Information Sheet	V1	21 October 2010
Participant Consent Form: Parent Consent Form	V1	21 October 2010
Participant Consent Form: Child Assent Form	V1	21 October 2010
Covering Letter		21 October 2010
Summary/Synopsis	V1	21 October 2010
Letter from Sponsor		26 October 2010
Clinical Details Proforma	V1	21 October 2010

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H1211/48	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Nicola Murphy
West Midlands REC Co-ordinator
For and on behalf of
Dr Matthew Dunn
Vice Chair

Email: nikki.murphy@westmidlands.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

This Research Ethics Committee is an advisory committee to West Midlands Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

11.2.3 Ethical Approval – Studies 5 and 6

National Research Ethics Service
NRES Committee North West – Haydock Park
North West Centre for Research Ethics Committees
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7819/7832
Facsimile: 0161 237 9427

28 April 2011

Dr Francis J Gilchrist
CF Research Fellow
University Hospital of South Manchester
Manchester Adult CF Centre
Wythenshawe Hospital
Southmoor Road
Manchester M23 9LG

Dear Dr Gilchrist

Study title:	**Exhaled breath hydrogen cyanide as a marker of Burkholderia cepacia complex in adults with cystic fibrosis**
REC reference:	**11/NW/0102**

Thank you for your letter of 26 March 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair (Professor Ravi Gulati – Consultant Physician) and Mrs Linda Ashcroft (Medical Statistician).

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter: Dr Francis Gilchrist, CF Research Fellow		16 February 2011
REC application	3.1	16 February 2011
Investigator CV: Dr Francis J Gilchrist		16 February 2011
Investigator CV: Professor Warren Lenney		
Participant Information Sheet: Control Group	1	08 February 2011
Participant Information Sheet: Bcc Group	1	08 February 2011
Participant Consent Form	1	08 February 2011
GP/Consultant Information Sheets	1	08 February 2011
Letter of invitation to participant: Control Group	1	08 February 2011
Letter of invitation to participant: Bcc Group	1	08 February 2011
Summary/Synopsis: Control Group	1	08 February 2011
Summary/Synopsis: Bcc Group	1	08 February 2011
Response to Request for Further Information: Dr Francis Gilchrist, CF Research Fellow		26 March 2011
Protocol	2	26 March 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email: -

referencegroup@nres.npsa.nhs.uk.

11/NW/0102	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Professor Ravi S Gulati
Chair

Email: noel.graham@northwest.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

11.3 ANNEX 3 - PRESENTATIONS RELEVENT TO PhD THESIS

Hydrogen cyanide concentrations in the breath of adult cystic fibrosis patients with and without *Pseudomonas aeruginosa* infection. Gilchrist FJ, Bright-Thomas RJ, Jones AM, Smith D, Španěl P, Webb AK, Lenney W

- European CF Conference, Lisbon, [Poster presentation] 12-15th June 2012 [J Cyst Fibros 2013; 12(Suppl 1):S83]

Is hydrogen cyanide a marker of *Burkholderia cepacia complex* infection? Gilchrist FJ, Sims H, Alcock A, Jones AM, Bright-Thomas RJ, Smith D, Španěl P, Webb AK, Lenney.

- British Thoracic Society Winter Meeting [Poster Presentation] 5-7th December 2012 [Thorax 2012; 67(Suppl 2):A102]

Exhaled breath hydrogen cyanide concentrations using Selected Ion Flow Tube Mass Spectroscopy (SIFT-MS); a comparison of on-line and off-line techniques. Gilchrist FJ, Razavi C, Jones AM, Webb AK, Spanel P, Smith A, Lenney W

- European CF Conference, Dublin, [Poster presentation] 7-9th June 2012 [J Cyst Fibros 2012; 11(Suppl 1): S84]

Quantification of 2-aminoacetophenone and hydrogen cyanide in the headspace of *Pseudomonas aeruginosa* cultured under biofilm and planktonic conditions by Selected Ion Flow Tube Mass Spectrometry. Gilchrist, FJ, Sims H, Alcock A, Belcher J, Jones AM, Smith D, Španěl P, Webb AK, Lenney W

- European Young CF Investigator Meeting [Oral and poster presentation] Paris. April 27th 2012. A, Lenney W

Difficulties and delays in obtaining NHS permission at the 8 centres involoved in The SPACE Study. Gilchrist FJ, Lenney W.

- NIHR Paediatric Specialities Group (Non-medicines). [Oral Presentation] London. 29th February 2012.

Variation in hydrogen cyanide production between different strains of *Pseudomonas aeruginosa.* Gilchrist FJ, Alcock A, Belcher J, Brady M, Jones AM, Smith D, Španěl P, Webb K, Lenney W.

- European Cystic Fibrosis Conference, [Poster Presentation] Hamburg. 10th June 2011. [J Cyst Fibros 2011; 10(Suppl 1): S40]
- Royal College of Paediatrics and Child Health Spring Meeting, [Oral Presentation] Warwick. 5th April 2011. [Arch Dis Child 2011;96:A14-15]
- Association of Paediatric Academics, [Oral Presentation] London. 17th December 2010.

11.4 ANNEX 4 - PUBLICATIONS RELEVENT TO PhD THESIS

Smith D, Španěl P, Gilchrist FJ, Lenney W. Hydrogen cyanide, a volatile biomarker of Pseudomonas aeruginosa. J Breath Res. 2013 Dec;7(4):044001.[209]

Gilchrist FJ, Sims H, Alcock A, Jones AM, Bright-Thomas RJ, Smith D, Španěl P, Webb AK, Lenney W. Is hydrogen cyanide a marker of Burkholderia cepacia complex? J Clin Microbiol. 2013 Nov;51(11):3849-51.[210]

Gilchrist FJ, Bright-Thomas RJ, Jones AM, Smith D, Spaněl P, Webb AK, Lenney W. Hydrogen cyanide concentrations in the breath of adult cystic fibrosis patients with and without Pseudomonas aeruginosa infection. J Breath Res. 2013 Jun;7(2):026010.[211]

Gilchrist FJ, Sims H, Alcock A, Belcher J, Jones AM, Smith D, Španěl P, Webb AK, Lenney W. Quantification of hydrogen cyanide and 2-aminoacetophenone in the headspace of *Pseudomonas aeruginosa* cultured under biofilm and planktonic conditions. Analytical Methods. 2012 Nov; 4(11):3661-65.[212]

Gilchrist FJ, Razavi C, Webb AK, Jones AM, Španěl P, Smith D, Lenney W. An investigation of suitable bag materials for the collection and storage of breath samples containing hydrogen cyanide. J Breath Res. 2012 Jul 4;6(3)036004.[213]

Gilchrist FJ, Alcock A, Belcher J, Brady M, Jones AM, Smith D, Španěl P, Webb K, Lenney W. Variation in hydrogen cyanide production between different strains of *Pseudomonas aeruginosa.* Eur Respir J 2011 Aug; 38:409-14.[214]

Lenney W, Gilchrist FJ. *Pseudomonas aeruginosa* and cyanide production. Eur Respir J 2011 Mar; 37(3):482-3.[215]