THE INFECTIVE PULMONARY EXACERBATION IN CYSTIC FIBROSIS:
AN ECOLOGICAL PERSPECTIVE

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Declaration of Originality

I declare that the work in this manuscript has been carried out by myself unless where stated.

The *Pseudomonas aeruginosa* hypermutator assays were performed by the Research Microbiology team, Papworth Hospital,

The Liverpool Epidemic Strain PA PCR was performed by the HPA Laboratory, Cambridge

The 16S rRNA gene sequencing was performed commercially by the Sanger Institute, Cambridge University. Preparation of raw sequencing data was performed by Dr Michael Cox, Department of Molecular Genetics and Genomics, NHLI, Imperial College.

The quorum-sensing assays were performed by Mr Nigel Halliday, School of Molecular Medicine, University of Nottingham.

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To the patients who agreed to take part in this study, without whose generosity this study would not have been possible.
ABSTRACT

Introduction

In CF, infective pulmonary exacerbations are common. Despite this, significant aspects of pathogenesis and patient responses to treatment remain poorly understood. This study examined a cohort of subjects receiving antibiotic therapy for exacerbations, correlated clinical findings with microbiological data and examined the factors leading to non-response to treatment.

Methods

Subjects were recruited when they commenced IV antibiotics. Measures of lung function, inflammatory markers and quality of life scores were collected with sputum samples during treatment. Samples were analysed for (i) routine microbiology, (ii) *Pseudomonas aeruginosa* (PA) hypermutator frequency (ii) 16S rRNA gene sequences to identify bacteria present, (iv) PA quorum-sensing (QS) molecule concentrations using LC-MS/MS.

Results

Patients who failed to return lung function to baseline at the end of treatment were shown to have a larger decline in FEV$_1$ at exacerbation and a longer time since stable measures. In treatment success, no significant improvement in lung function was seen after 7 days treatment. No association was seen between severity of exacerbation or treatment response and PA mucoidy or presence of *Staphylococcus aureus*. Hypermutator PA was seen more in older patients and associated with milder exacerbations. Bacterial diversity was shown not to change significantly during antibiotic treatment but there was a significant relationship between the change in diversity and change in PA bacterial density. Meropenem was shown to have greater effect on diversity than ceftazidime. A significant association between QS molecule concentration and lung function decline at exacerbation was shown. QS concentrations were seen to decrease significantly during antibiotic treatment which was not associated with a decline in bacterial numbers.

Discussion

Current durations of antibiotic treatment may not be appropriate as most improvement is seen within 7 days. The effect of repeated course of meropenem on bacterial diversity requires investigation. QS inhibition may provide alternative therapeutic options to exacerbation treatments.
### ABBREVIATIONS USED IN THIS THESIS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-oxo-C12-AHL</td>
<td>N-(3-oxo-dodecanoyl)-L-homoserine lactone</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway Surface Layer</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri-phosphate</td>
</tr>
<tr>
<td>BCFQ</td>
<td>Brompton Cystic Fibrosis Questionnaire</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>C4-AHL</td>
<td>N-butanoyl-homoserine lactone</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>CFQ-R</td>
<td>Cystic Fibrosis Questionnaire - Revised</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive Protein</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium (Na) Channel</td>
</tr>
<tr>
<td>FEF&lt;sub&gt;25,75&lt;/sub&gt;</td>
<td>Forced Expiratory Flow (mean flow between 25 and 75% of remaining FVC)</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced Expiratory Volume in 1 second</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; %OBL</td>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; as a percentage of the stable baseline value</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>HAQ</td>
<td>4-hydroxy-2-alkylquinolones</td>
</tr>
<tr>
<td>HHQ</td>
<td>4-hydroxy-2-heptylquinolone</td>
</tr>
<tr>
<td>HQNO</td>
<td>2-heptyl-4-hydroxyquinolone N-oxide</td>
</tr>
<tr>
<td>IECF</td>
<td>Infective Exacerbation of CF</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LES</td>
<td>Liverpool Epidemic Strain</td>
</tr>
<tr>
<td>MARPA</td>
<td>Multiple Antibiotic Resistant <em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>MHA</td>
<td>Mueller-Hinton Agar</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational Taxon Unit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PA</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>PC1</td>
<td>Principal Co-ordinate 1</td>
</tr>
<tr>
<td>PCL</td>
<td>Peri-ciliary Layer</td>
</tr>
<tr>
<td>PCoA</td>
<td>Principle Co-ordinate Analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PQS</td>
<td>2-heptyl-3,4-dihydroxyquinolone</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>QOL</td>
<td>Quality of Life</td>
</tr>
<tr>
<td>QS</td>
<td>Quorum Sensing</td>
</tr>
<tr>
<td>RBH</td>
<td>Royal Brompton Hospital</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
</tr>
<tr>
<td>SA</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>TRFLP</td>
<td>Terminal Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>TTNE</td>
<td>Time to Next Exacerbation</td>
</tr>
<tr>
<td>WCC</td>
<td>White Cell Count</td>
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</table>
# TABLE OF CONTENTS

## Chapter 1 – Introduction

1.1 What is Cystic Fibrosis? ................................................................. 14
1.2 Functions of the CFTR protein and the cellular basis for airway disease .......... 17
1.3 Microbiology of CF airway disease .................................................. 20
1.4 Acute respiratory infections in CF – the pulmonary infective exacerbation .......... 26
1.5 What causes an acute pulmonary exacerbation in CF? ................................ 30
1.6 *Pseudomonas aeruginosa* ................................................................ 31
1.7 Quorum Sensing Systems in *Pseudomonas aeruginosa* .............................. 36
1.7 Hypermutator Strains of *Pseudomonas aeruginosa* ................................... 38
1.8 Study Hypotheses ............................................................................. 40
1.9 Plan of Investigation ......................................................................... 40

## Chapter 2 – The CF Infective Exacerbation – A Clinical Tracking Study

2.1 Introduction ..................................................................................... 42
2.2 Methods ......................................................................................... 42
2.3 Results ........................................................................................... 48
2.4 Discussion ....................................................................................... 72

## Chapter 3 – The CF Infective Exacerbation – A Microbiological Study

3.1 Introduction ..................................................................................... 80
3.2 Methods .......................................................................................... 81
3.3 Results ........................................................................................... 84
3.4 Discussion ....................................................................................... 96
Chapter 4 – Hypermutator strains of Pseudomonas aeruginosa – their Role in CF Exacerbations

4.1 Introduction ........................................................................................................... 102
4.2 Methods .................................................................................................................. 103
4.3 Results ..................................................................................................................... 105
4.4 Discussion ............................................................................................................... 109

Chapter 5 – The CF Infective Exacerbation – A Microbiome Analysis using 16S rRNA Gene Sequencing

5.1 Introduction .............................................................................................................. 112
5.2 Methods .................................................................................................................. 113
5.3 Results ..................................................................................................................... 118
5.4 Discussion ............................................................................................................... 139

Chapter 6 – Quorum sensing by Pseudomonas aeruginosa in CF exacerbations

6.1 Introduction .............................................................................................................. 143
6.2 Methods .................................................................................................................. 144
6.3 Results ..................................................................................................................... 147
6.4 Discussion ............................................................................................................... 160

Chapter 7 – Discussion and Plans for Future Study ........................................................... 166

References .................................................................................................................... 174

Appendix ....................................................................................................................... 186
FIGURES

Figure 1.1 UK CF Registry data demonstrating the changing bacterial infections with age in the UK CF population. UK CF Registry Data 2009
Figure 2.1 The % change in FEV\textsubscript{1} during treatment of an exacerbation. A comparison of subjects receiving ceftazidime with subject receiving meropenem
Figure 2.2 The % change in FEV\textsubscript{1} during treatment of an exacerbation. A comparison of subjects receiving 10 days of treatment with those receiving 14 days
Figure 2.3 A comparison of time to next exacerbation values in subjects receiving meropenem or ceftazidime
Figure 2.4 A comparison of time to next exacerbation values in subjects receiving 10 or 14 days of antibiotic therapy
Figure 2.5 The change in FEV\textsubscript{1}, FEV\textsubscript{1} % predicted and FEV\textsubscript{1} %OBL during treatment
Figure 2.6 FEV\textsubscript{1} for all patients at each time point with treatment success subjects in blue and treatment failure subjects in red
Figure 2.7 FEV\textsubscript{1} % predicted for all patients at each time point with treatment success subjects in blue and treatment failure subjects in red
Figure 2.8 FEV\textsubscript{1} as a percentage of baseline for all patients at each time point
Figure 2.9 A comparison of the changes in FEV\textsubscript{1}, FEV\textsubscript{1} % predicted and FEV\textsubscript{1} %OBL during treatment by treatment outcome groups
Figure 2.10 Individual CRP responses to antibiotic therapy
Figure 2.11 Individual total white cell count responses to antibiotic therapy
Figure 2.12 Individual neutrophil count responses to antibiotic therapy
Figure 3.1 Day 1 inflammatory markers comparing non-mucoid and mucoid morphotypes of PA
Figure 3.2 (a) Day 1 spirometry values comparing non-mucoid and mucoid morphotypes of PA (b) Change in spirometry comparing morphotypes
Figure 3.3 PA CFU/ml prior to commencing therapy comparing treatment outcomes
Figure 3.4 PA bacterial numbers during treatment and comparing treatment outcome groups
Figure 3.5 The correlation between change in FEV\textsubscript{1}(L) and the change in PA cfu/ml during treatment
Figure 3.6  The correlation between change in FEV$_1$ as a percentage and change in PA cfu/ml during antibiotic treatment.........................91

Figure 3.7  Changes in PA bacterial density grouped by whether the antibiotic sensitivity profile of the bacterial was resistant to one or more of the antibiotic therapies given ..................................................93

Figure 3.8  Graphs representing median values of spirometry measures in groups defined by the presence or absence of SA on bacterial culture.........................94

Figure 4.1  Individual changes in FEV1 %predicted between the start and end of treatment.................................................................106

Figure 4.2  A box and whisker plot comparing the change in PA bacterial density during antibiotic therapy between groups defined by the presence or absence of hypermutator strains of PA .......................................108

Figure 5.1  Relative Abundance of OTU’s on day 1 of treatment for each subject ..........120

Figure 5.2  Principal co-ordinate analysis demonstrating clustering of samples by subject.............................................................................121

Figure 5.3  Bi-plot of PCoA of variance in weighted Unifrac distances overlaid with taxa data........................................................................122

Figure 5.4  Species richness at each measured time point of the exacerbation..............126

Figure 5.5  Pielou’s evenness index at each measured time point of the exacerbation ....126

Figure 5.6  Shannon diversity index at each measured time point of the exacerbation ....127

Figure 5.7  A comparison of species richness in treatment outcome groups at each measured time point of an exacerbation treatment...............................129

Figure 5.8  A comparison of Pielou’s evenness indices in treatment outcome groups at each measured time point of an exacerbation treatment .........................129

Figure 5.9  A comparison of Shannon diversity indices in treatment outcome groups at each measured time point of an exacerbation treatment .......................130

Figure 5.10  The correlation between change in PA bacterial density (cfu/ml) over the treatment course and the change in the Pielou’s evenness index .................132

Figure 5.11  The correlation between change in PA bacterial density (cfu/ml) over the treatment course and the change in the Shannon diversity index ...............133

Figure 5.12  (a) Species richness during antibiotic therapy comparing those subjects receiving meropenem with those receiving ceftazidime. Data presented as median values.................................................................135

(b) Species richness during antibiotic therapy comparing those subjects receiving meropenem with those receiving ceftazidime. Data presented as individual subjects........................................................................135
Figure 5.13  Pielou’s evenness index during antibiotic therapy comparing those subjects receiving meropenem with those receiving ceftazidime ..................136

Figure 5.14  Shannon diversity index during antibiotic therapy comparing those subjects receiving meropenem with those receiving ceftazidime .................136

Figure 6.1  Median concentrations of QS analytes on day 1 of the study ..................148

Figure 6.2  Median QS analyte concentrations in sputum samples grouped by the presence or absence of mucoid PA .................................................................155

Figure 6.3  Median QS analyte concentrations in sputum samples grouped by the presence or absence of hypermutator strains of PA ..............................................156

Figure 6.4  QS analyte concentrations during antibiotic therapy ..........................158
### TABLES

**Table 2.1** Baseline characteristics of study subjects. All subjects and then a comparison of groups cohorted by treatment outcome ........................................49

**Table 2.2** Spirometry values during a CF exacerbation ..........................................................56

**Table 2.3** Median changes in lung function during exacerbation treatment and comparing groups defined by treatment outcomes ........................................57

**Table 2.4** CRP median values during antibiotic therapy and comparison of treatment groups ........................................................................................................64

**Table 2.5** Median total white cell counts and neutrophil counts during exacerbation treatment and a comparison of groups cohorted by treatment outcome ..........66

**Table 2.6** Correlations between spirometry and white cell counts and between inflammatory markers and time to next exacerbation ........................................69

**Table 2.7** Median BMI values at the start and end of exacerbation treatment and a comparison of groups cohorted by treatment outcome ........................................70

**Table 2.8** CFQ-R respiratory domain score during treatment and comparing treatment outcome groups ........................................................................................................71

**Table 2.9** BCFQ score during treatment and comparing treatment outcome groups............71

**Table 3.1** Day 1 spirometry values and inflammatory markers comparing non-mucoid and mucoid morphotypes of PA. Changes in spirometry during treatment comparing non-mucoid and mucoid morphotypes of PA ..........85

**Table 3.2** Correlations between day 1 spirometry values and PA bacterial numbers (cfu/ml)..........................................................................................................................87

**Table 3.3** The change in PA bacterial numbers during treatment and comparing treatment outcome groups ........................................................................................................89

**Table 3.4** Correlations comparing changes in spirometry with changes in PA bacterial numbers ....................................................................................................................90

**Table 3.5** The effect of antibiotic sensitivity on clinical and bacterial factors ......................92

**Table 3.6** The clinical effect and associations with isolation of *Candida albicans* in sputum culture .........................................................................................................................95

**Table 4.1** A comparison of clinical measures on day 1 of antibiotic treatment in groups defined by the presence or absence of hypermutator strains of PA ....105

**Table 4.2** A comparison of PA bacterial density at the beginning and end of antibiotic therapy and the median change in bacterial density in groups defined by the presence or absence of hypermutator strains of PA ..........107

**Table 5.1** Relative abundance of the top 20 OTU’s for all samples ..............................119

**Table 5.2** Correlations between bacterial diversity indices and spirometry values .......124
Table 5.3  Diversity indices during antibiotic therapy and a comparison with day 1 of treatment values ........................................................................................................125
Table 5.4  Diversity indices at each measured time point comparing treatment outcome groups ........................................................................................................128
Table 5.5  Correlations between the change in bacterial diversity indices and the change in spirometry over the treatment course ........................................131
Table 5.6  Correlations between the change in PA bacterial density and the change in bacterial diversity indices .................................................................132
Table 5.7  Bacterial diversity indices in cohorts divided by beta-lactam antibiotic received during treatment ..................................................................................134
Table 5.8  Bacterial diversity indices in the presence and absence of PA hypermutator strains ........................................................................................................137
Table 5.9  Correlations between bacterial diversity indices at the end of antibiotic therapy and time to next exacerbation values ........................................138
Table 6.1  Detection of QS analytes in samples and median ages ........................................147
Table 6.2  Median day 1 concentrations of the QS analytes ................................................148
Table 6.3  Correlations between subject age and QS analyte concentrations on day 1 of the clinical study ..............................................................................149
Table 6.4  Correlations between QS analyte concentrations and spirometry measures on day 1 of the clinical study ........................................................................150
Table 6.5  Correlations between QS analytes and inflammatory markers on day 1 of treatment ........................................................................................................151
Table 6.6  QS analyte concentrations at the beginning and end of treatment comparing treatment outcome groups .................................................................152
Table 6.7  Median QS analyte concentrations in sputum samples grouped by the presence or absence of mucoid PA .................................................................153
Table 6.8  Comparing QS analyte concentrations by the presence or absence of PA hypermutator strains .................................................................154
Table 6.9  QS analyte concentrations at each time point of the study .......................................157
Table 6.10 Correlations between PA bacterial density and QS analyte concentration on day 1 of treatment and between the change in PA density and the change in QS analyte concentration over the course of antibiotic therapy .....159
CHAPTER 1
INTRODUCTION

1.1 What Is Cystic Fibrosis?

Cystic Fibrosis (CF) is an inherited multi-system disorder and is the commonest autosomal recessive condition in Caucasian populations with 1 in 25 people carrying a disease-causing mutation. It is characterised by bronchiectasis and disorders of the bowel and pancreas including pancreatic insufficiency, CF-related diabetes and liver disease.

Where once it was a disease that led to death in early childhood from bowel or respiratory complications, intensive treatments, driven by dedicated scientific research, have led to great improvements in survival rates and patient morbidity. In the UK, median life expectancy has improved rapidly over the past few decades and is currently 41.5 years (2011 registry data).

The story of the discovery of CF as a condition and the subsequent experimental data that led to diagnostic tests and then the underlying genetic cause is well documented.

The term “Cystic Fibrosis” was first used in the 1930’s by an American pathologist, Dr Dorothy Andersen\(^1\). She reported on a case series of children with bowel disease and bronchiectasis and collected clinical histories and data from detailed autopsy studies on 49 patients. While others had previously noted an association between pancreatic disease and bronchiectasis, she was the first to compile a complete description of CF as a distinct disease. “Cystic Fibrosis” is actually the term used to describe characteristic pathological changes seen in the pancreas of the cohort group.

Dr Andersen was also the first to note the autosomal recessive nature of CF as an inheritable disease\(^2\).
In the 1950s, during a heat wave on the Eastern Seaboard in the United States, the majority of children admitted with heatstroke to New York hospitals had CF\textsuperscript{3}. Another New York paediatrician, Paul di Sant’Agnese investigated the cause of this and discovered that the sweat of these patients contained high levels of sodium chloride\textsuperscript{4}, an observation that led to the first diagnostic tests for CF, the sweat sodium and sweat chloride tests. In the ensuing decades, survival rates for CF patients improved due to the introduction of antibiotics and a greater understanding of the increased nutritional needs of CF patients. In 1983, Quinton reported on a series of experiments showing that the underlying cause for the abnormal sweat test was chloride impermeability, thus giving the first description of the underlying cellular defect as specifically related to chloride channels\textsuperscript{5}. Later in the 1980s, with the rise in genetic research, an intensive search was made to discover the gene whose mutation was responsible for CF. Tsui et al were the first to locate it at the chromosomal level, to the long arm of chromosome 7\textsuperscript{6}. Within a few years of this discovery, the gene responsible for CF was accurately located, characterised and termed the cystic fibrosis transmembrane conductance regulator (\textit{CFTR}) gene\textsuperscript{7-9}. The \textit{CFTR} gene is located in region q31.2 on the long arm of chromosome 7. It contains approximately 250,000 base pairs with 27 exons and codes for a protein of 1480 amino acids, known as the \textit{CFTR} protein. This protein is part of a family known as ATP-binding cassette (ABC) transporters. It is a trans-membrane protein that transports chloride ions across the cell membrane under the regulation of cAMP signalling and is located predominantly in the apical membrane of epithelial cells\textsuperscript{10}.

Since the discovery of the \textit{CFTR} gene nearly two thousand mutations have been noted to cause a defect in \textit{CFTR} protein synthesis, although not all of these have been confirmed as causing CF.

The commonest mutation is a deletion of 3 base pairs at codon 508 (Phe508del). It accounts for approximately two thirds of all CF mutations. Worldwide, only 4 other mutations have
frequencies greater than 1% among CF chromosomes (G542X, N1303K, G551D, and W1282X).\textsuperscript{11}

Mutations of the CFTR gene have been classified according to their effect on CFTR function.\textsuperscript{12}

Class I mutations prevent production of full-length CFTR protein because of nonsense or frameshift mutations, or because of aberrant splicing of mRNA due to stop codon-inducing mutations. A common example of this is G542X.

Class II mutations affect protein maturation. Such mutations cause misfolding of the CFTR protein leading to an inability of the protein to transfer to the cell membrane; Phe508del is an example of this.

Class III mutations affect chloride channel regulation. In these mutations CFTR is transferred to the cell surface but then does not respond appropriately to cAMP stimulation, remaining closed for most of the time. G551D is an example of such a mutation.

Class IV mutations affect chloride ion conductance. The CFTR protein is in the correct place in the cell membrane, responds appropriately to cAMP stimulation but chloride current is reduced. An example would be the R117H mutation.

Class V mutations affect protein stability. Truncated CFTR proteins produced by this mutation act normally but have a much reduced half-life in the cell membrane. An example of this is the Q1412X mutation.

It is clear from clinical practice that the knowledge of a patient’s gene mutations does not allow us to predict with certainty their clinical outcome. While some mutations are associated with generally mild phenotypes, even those associated with potentially more deleterious effects such as Phe508del have a wide range of phenotypes from severe disease to being
relatively asymptomatic. A study of long term survivors (those reaching the age of 40 years) showed that 30% of patients were homozygous for Phe508del.\textsuperscript{13}

It is unlikely that the CFTR mutation alone can fully explain the CF disease process, and it may be that other genes, so called modifier genes, may also contribute to the expression of the CF phenotype which is an area of ongoing study.

1.2 Functions of CFTR Protein and Cellular Basis for Airway Disease

The CFTR protein acts as a chloride ion transporter, mainly at the apical surface of epithelial cells. However CFTR is found in a wide number of different cells and may be involved in many other intracellular processes not directly related to ion transport.

The CFTR Protein as an Ion Transporter

The CFTR protein is one of many ion channels that regulate ion and water transportation across the airway epithelium. A number of studies have examined the cellular mechanisms that occur in healthy lung and the effect that CF has on these processes is summarised in a review by Boucher\textsuperscript{14}. Using these data, a hypothesis known as the low volume airways surface liquid theory is the most widely accepted model for the cause of airway disease in CF\textsuperscript{15}.

The apical surface of the airway epithelial cells is ciliated. A layer of fluid, known as the airway surface layer (ASL), bathes these cilia. The ASL is composed of two layers. The peri-ciliary layer (PCL) abuts the epithelial cell surface and contains fluid that bathes the cilia. Lying above this is the mucus layer which is continuously moved by the cilia to transport debris and bacteria out of the lungs. This mucus layer can also act as a reservoir of fluid for the peri-ciliary layer. In healthy lungs the epithelial cells have an ability to both deplete and add fluid to the PCL layer.
Both the apical surface and basolateral surface of the epithelial cells have ion transporters to regulate fluid volume within the ASL. In healthy tissue an electrochemical gradient is generated across the epithelium by a basolateral Na\(^+\)/K\(^+\)/ATPase pump. This attracts Na\(^+\) ions from the airway surface liquid to the interstitium. In the resting state the major ion transport process is active Na\(^+\) absorption which is regulated by an apical membrane epithelial Na channel (ENaC). In this resting state chloride is transported via a paracellular pathway to maintain electrochemical neutrality with Na\(^+\) ions and water follows Na\(^+\) and Cl\(^-\) via cell membrane channels (aquaporins).

CFTR is active when the ASL is dehydrated. The major activity of CFTR in healthy tissue is inhibition of ENaC, preventing Na\(^+\) entering the intracellular space. It appears that this is occurring at all times to a greater or lesser extent, so called tonic inhibition. CFTR also acts to secrete Cl\(^-\) ions into the ASL which leads to a net movement of water into the ASL thus rehydrating the PCL and mucus layers.

When the CFTR protein is absent or dysfunctional, as in CF, this regulation of ASL fluid is lost. The lack of ENaC inhibition leads to hyperabsorption of Na\(^+\) ions with a net movement of water out of the ASL. Equally Cl\(^-\) secretion into the ASL is markedly decreased (some occurs via an alternative chloride channel, the calcium-activated chloride channel). The overall effect is to dehydrate the PCL and mucus layers leading to viscous, sticky mucus coating the epithelial cilia. This leads to a loss of effective mucus clearance and subsequent risk of bacterial infection.

While this low volume hypothesis is currently the favoured hypothesis it is unlikely that this defect alone causes the clinical picture we see. For example, investigators have shown that the introduction of a shear stress through use of a phasic motion to CF cell cultures can release sufficient ATP to decrease Na\(^+\) absorption and activate alternative Cl\(^-\) channels so as to
return the PCL to a physiological depth. The same authors identified that a “second-hit” to the CF epithelia of a viral infection such as respiratory syncytial virus (RSV) may activate hydrolysis of ATP and as such ameliorate the effect of the shear stress and lead to depletion of the PCL volume\textsuperscript{16}.

**Non-Ion Transport Functions of the CFTR Protein**

While the original discovery of the underlying pathology of CF and indeed the identification of the CFTR protein as a transmembrane channel, would suggest the primary function of the CFTR protein is that of an ion transporter channel it is clear from a large number of studies that CFTR has other roles within human cells including glycosylation and sialylation of glycoproteins\textsuperscript{17}, and effects on bicarbonate secretion\textsuperscript{18}.

It is also likely that CFTR dysfunction has an effect separate to ion transportation that leads to upregulation of the inflammatory pathway which may be exaggerated by the presence of bacteria\textsuperscript{19}. A discussion of these mechanisms is beyond the scope of this report but include upregulation of NF-kB and resultant over-expression of pro-inflammatory cytokines, an effect of the unfolded CFTR protein on the endoplasmic reticulum leading to disorders of intracellular calcium homeostasis, and the action of CFTR as a transporter of the anti-oxidant, glutathione.
1.3 Microbiology of CF Airway Disease

The loss of effective mucociliary clearance in the CF airway leads to infection with inhaled pathogens such as bacteria, viruses and certain fungi. Pulmonary infection is the leading cause of morbidity and mortality in CF and as such, a clear understanding of the processes involved in the acquisition and treatment of infections is paramount in treating the CF patient.

Bacterial Flora

A wide variety of organisms can infect the CF airway, often initially common pathogens such as *Staphylococcus aureus* (SA) and *Haemophilus influenza* (HI) and then, at a later stage, opportunistic bacteria such as *Pseudomonas aeruginosa* (PA) and *Burkholderia sp.* A number of investigators have examined the changing nature of respiratory pathogens in CF, looking both at how changes in individual patients have occurred and also the population in general with the emergence of new pathogens.

Prior to the introduction of antibiotics to medical practice, the commonest pathogen isolated from CF patients was SA. This is likely to represent the young age at which CF patients died in that era as the most recent US registry data suggests that in children and adolescents SA remains the commonest respiratory pathogen isolated. This organism is a commensal of human skin and is commonly isolated from the anterior nares of asymptomatic healthy people. It therefore has a large community reservoir from which the CF patient can acquire the organism. A recent study has highlighted that the family members of CF children with SA infection are far more likely to carry an identical strain in their noses, thus providing a source or common exposure to the infecting organism.

Effective antibiotic therapy for SA and prolonged life spans has led to the emergence of other respiratory pathogens. Mearns et al. examined changing patterns of CF airway microbiology from the 1950s through to 1971. She noted a decrease in SA infection from 86% in 1950-
57, to 30% in 1969-71 in children of less than 1 year of age. Overall for her clinic, she noted a
decrease in SA isolation from 45% to 12% and an increase in PA from 3 to 28%. It was also
noted that the largest decrease in SA infection and the greatest increase in PA infection was
seen in those with the most severe lung disease. Mearns postulated that infection with PA
was a marker of disease severity rather than the cause of the damage seen.

A more recent adult cohort was examined in a single centre for the years 1985-2005. Millar
et al\textsuperscript{23} noted the commonest organism isolated from the patient group was PA, the incidence
of which remained stable over the study period (77-82%). The incidence of SA infection also
remained relatively stable (54- 47%). The incidence of \textit{Haemophilus influenzae}, \textit{Aspergillus}
and \textit{Burkholderia cepacia} complex all decreased while \textit{Stenotrophomonas maltophilia}
increased. It was noted that the incidence of methicillin-resistant \textit{S. aureus} (MRSA) also
increased (1% to 4%) over the study period.

While these studies are only related to single centres, the introduction of national CF
databases has allowed us to examine respiratory pathogens on a much larger scale. Both the
UK and US CF databases confirm that PA is the commonest respiratory pathogen in adults. In
the UK data, from the 2009 report, 67% of CF patients aged 28-31 are infected with the
organism, (Figure 1.1). The US data registry generally concurs with this data although
records much higher rates of MRSA infection (23.7% of all CF patients, adult and paediatric).
More recently investigators have examined the role of anaerobic bacteria in CF infections. These bacteria are often overlooked as they are not routinely cultured. A number of studies have confirmed that anaerobic bacteria are present in significant numbers in CF sputum samples. Worlitzsch et al\textsuperscript{24} identified 35 different species of anaerobic bacteria in 114 CF sputum samples, with a maximum number of 4 species in one sample. The authors also noted that of the samples taken at the time of an exacerbation, 58\% of anaerobic bacteria were resistant \textit{in vivo} to the prescribed antibiotics. Some would argue that the anaerobes present represent oro-pharyngeal contamination but significant numbers of anaerobic bacteria in broncho-alveolar lavage fluid, would suggest against this\textsuperscript{25}. As anaerobic bacteria are commensal organism in the oro-pharynx, is there evidence they are pathogenic in the lungs? An editorial by AM Jones\textsuperscript{26} succinctly summarises the arguments regarding this discussion and concludes that the evidence for pathogenicity is not sufficient to warrant a change in current antibiotic practices.
Non-Culture Techniques for Bacterial Identification

In clinical practice, current laboratory methods for identifying bacteria in sputum involve the culturing of sputum samples on selective media to determine bacterial growth. This process is relatively slow and, perhaps more importantly, by using selective media a full picture of bacterial biodiversity within the CF lung may not be appreciated.

Bacterial identification by genetic sequencing is not a particularly new science, but recent technological advances have allowed large amounts of genetic material to be sequenced in relatively short time frames and much more economically. Such techniques involve the analysis of the 16S ribosomal RNA (rRNA) gene. The 16S rRNA gene codes for the small subunit of the bacterial ribosome (16S rRNA is only seen in prokaryotic organisms, eukaryotes have an 18S rRNA gene). The 16S rRNA gene is ideal for identification as it is constant between bacterial species and is relatively short (1.5K base pairs) allowing full sequencing. Such techniques do not require the sputum sample to be incubated and cultured on selective media allowing a far more accurate analysis of the diversity of the bacteria that are present in the CF lung. In the CF literature, 2 main forms of bacterial identification have been used; (i) terminal restriction fragment length polymorphism (T-RFLP) analysis, (ii) sequencing of the 16S rRNA gene.

Both methods involve the extraction of bacterial DNA from clinical samples followed by amplification of the 16S rRNA gene using a polymerase chain reaction (PCR) and primers directed at the appropriate gene. In T-RFLP analysis, the amplified gene products (amplicons) are then digested with a restriction endonuclease enzyme. This fragments the amplicons into variable sizes dependent on the position of the restriction site which differs between bacterial species. These fragments can then be separated by gel electrophoresis and the presence of bacterial species can be determined by fragment size. T-RFLP is limited by the resolution of
the data; many bacterial species have similar T-RFLP fragment lengths and it can be difficult to distinguish separate species in some cases. Gene sequencing allows a much higher resolution of the data and gives a more accurate picture of the level of microbiological diversity seen in clinical samples.

The commonest form of gene sequencing used in microbiology is pyrosequencing. Essentially this process involves the sequencing of a single strand of DNA (amplicon) by sequentially adding nucleotides in the presence of the enzymes, DNA polymerase, ATP sulfurylase and a luciferase (light-emitting enzyme). When a complementary base is added pyrophosphate is released from the DNA polymerase. This is then converted to ATP by ATP sulfurylase, activating the luciferase which leads to a detectable light emission. Any remaining nucleotide is removed with apyrase before the next nucleotide is added. By comparing the pattern of light emissions with the nucleotide added it is possible to determine the DNA sequence.

Early work examining the CF lung microbiome utilised T-RFLP. One of the first studies, performed by Rogers et al, analysed 71 CF sputum samples. They revealed the presence of 19 species of bacteria including 15 never before documented in CF infection and a significant number of strict anaerobes. They determined mean species richness (the number of bacteria per sample) of 13.3 distinct T-RF bands, clearly establishing the CF lung as a complex microbiological environment with a far more diverse population of bacteria than previously thought.

Armed with more in-depth knowledge of the true diversity of bacteria in the CF lung it is interesting to postulate what clinical effect this could have. For example, a condition such as *Clostridium difficile* diarrhoea is due to an overgrowth of the bacteria in an environment which usually has a diverse range of bacteria. It is therefore possible that alterations to the
bacterial diversity within the CF lung, with selective growth of one virulent bacterial species could have similar pathological effects.

Certainly it appears that bacterial diversity does change with age. A small study of a cross section of CF patients of all ages noted an increase in bacterial diversity during the first decade of life followed by a gradual decrease in diversity as patients were older. The study is limited by its small number of patients and its cross sectional nature and further work is required to determine any link between bacterial diversity and disease progression and severity.

Two studies have examined the changes in diversity of bacteria in CF sputum during treatment of an exacerbation. In a paper by Tunney et al the authors found that bacterial diversity between patients was large but that antibiotic therapy did not significantly affect diversity. However the study was limited by the small number of patients, the wide range of antibiotics used and the use of T-RFLP, which may have underestimated the diversity of organisms present. A more recent paper by Daniels et al, also examined the CF exacerbation. They found that relative PA abundance increased during antibiotic therapy with a decrease in the number of non-pseudomonal species detected. This study also used T-RFLP and included patients receiving both oral and intravenous antibiotic courses. A further longitudinal study by Zhao et al, followed 6 CF patients for up to 9 years with regular sputum collection. These samples were then analysed with 16s rRNA pyrosequencing. While this study was not designed to look at exacerbations per se, they did record the clinical status of the patient at the time of sputum collection and some samples were taken at the time of exacerbation. They found that bacterial diversity decreased over time in patients who had a progressive decline in lung function but remained stable in those with no significant progression of their lung disease. They also found that exacerbations did not significantly
change the community structure but antibiotic treatment was associated with a decrease in bacterial diversity, with recovery of diversity following the cessation of antibiotics.

There does appear to be some contradiction in these exacerbation studies that requires further examination using prospectively gathered data with clearly defined exacerbations and treatment protocols. Clearly patients are improving in terms of symptoms, increases in lung function, and reductions in inflammatory markers. It remains unclear therefore whether bacterial diversity is important or whether we need to examine more closely the role of intra-species diversity such as the relative abundance of quorum-sensing proficient PA organisms, which will be discussed further.

1.4 Acute Respiratory Infections in CF

Respiratory infections are responsible for the bulk of morbidity and mortality in CF. They cause a chronic decline in lung function which eventually leads to death from respiratory failure. Initial infection with SA or PA may be relatively asymptomatic and as such, regular sputum cultures are required as a surveillance tactic to pick up new infections and to attempt eradication of the organism before chronic infection can be established. However, the bulk of treatment for infections in CF patients is for acute exacerbations of chronic infection. Clearly, understanding the trigger for these exacerbations and the response of the organisms to treatment should allow optimisation of therapy.

Data from the UK CF registry showed that 50% of CF patients received a course of IV antibiotics over a one year period with the mean total duration of antibiotic treatment over a year of 28 days\textsuperscript{20}. These figures vary with age, being highest in the age group 24-27 years where 65% of patients received IV antibiotics for a mean of 31 days.
This figure is really only the tip of iceberg when it comes to the treatment burden of CF infection. Many acute exacerbations are treated successfully with oral antibiotics. Equally chronic infections with some organisms require long-term inhaled and/or oral antibiotics in an attempt to suppress bacteria and prevent acute infective exacerbations.

**Defining an Infective Exacerbation**

While it may seem a simple task, defining what constitutes an acute infective exacerbation has generated a large amount of discussion in the CF medical community. Many studies have adopted a Cartesian philosophy, suggesting that an exacerbation exists when an attending clinician says it does. This is problematic as it has clearly been demonstrated that a lack of consensus exists between clinicians as to what constitutes an infective exacerbation.

As yet there are no fixed criteria for what defines an infective exacerbation. Perhaps the closest we have are the criteria produced following the 1994 Cystic Fibrosis Foundation Microbiology and Infectious Disease Consensus Conference.

This requires the presence of at least three of the following eleven new findings or changes in clinical status when compared to the most recent baseline visit:

1. Increased cough
2. Increased sputum production, change in appearance of sputum, or both
3. Fever on more than one occasion in the previous week
4. Weight loss greater than or equal to 1kg or 5% of body weight associated with anorexia and decreased dietary intake
5. School or work absenteeism in the previous week
6. Increased respiratory rate, increased work of breathing or both
7. New findings on chest examination
8. Decreased exercise tolerance
9. Decrease in FEV1 of greater than or equal to 10 % from previous baseline study (defined as best outpatient FEV1 in past 12 months)
10. Decrease in oxygen saturation from baseline value within three months of >10%
11. New finding on chest radiograph

A number of other criteria have been used in CF studies. These include the Fuchs criteria\textsuperscript{34} and criteria determined by Ramsey et al. for the inhaled TOBI trial\textsuperscript{35}, and are broadly similar to the 1994 consensus criteria described above.

Rosenfeld et al describe a multivariate model to create an algorithm identifying patients with a pulmonary exacerbation. They determined that clinical signs and symptoms had a greater predictive value than any laboratory measured values. Most predictive were increased cough, change in sputum volume or consistency, decreased appetite/weight, change in respiratory examination and respiratory rate\textsuperscript{36}. The authors produced a scoring system known as the PEx score. However this has not been significantly used or validated in other clinical trials.

**Treatment of Infective Pulmonary Exacerbations**

The majority of CF infective pulmonary exacerbations are treated with antibiotics directed at the organism that chronically infects the airways. For many bacteria the choice of antibiotics is a simple one. However, given the virulence factors of PA and its ability to rapidly develop antibiotic resistance, choosing appropriate antibiotics to treat this organism has been extensively studied.

Only one class of oral antibiotics, the quinolones, are recognised to have activity against PA. Unfortunately resistance to ciprofloxacin (the most commonly used oral quinolone) has been reported as high as 30\% in the UK\textsuperscript{37}. Because of the relative lack of good oral treatments patients often require intravenous antibiotics as a first line treatment for PA-induced infective exacerbations.

A significant number of intravenous antibiotics have activity against PA. These included aminoglycosides such as gentamicin and tobramycin, polymixins such as colistin sulphate,
and β-lactam based antibiotics (those containing a β-lactam ring) such as penicillins (temocillin, piperacillin), carbepenems (imipenem, meropenem) and some cephalosporins (ceftazidime).

Current guidelines recommend combination antibiotic therapy\(^3\)\(^8\). Most patients will therefore receive a β-lactam-based antibiotic in combination with an aminoglycoside. The reasoning behind this decision is that of preventing the development of antibiotic resistance. While this is a theoretical concern, no study has shown this to occur. A number of small studies comparing antibiotic monotherapy with combination therapy have shown no significant increase in the development of resistance and similar clinical outcomes for both treatment groups\(^3\)\(^9\)\(^4\)\(^6\). It could be argued that the serious side effects of aminoglycosides such as renal and oto-toxicity, present a greater risk than the benefit of perceived decrease in antibiotic resistance. The most recent American CF Foundation guidelines suggest further work needs to be undertaken in this area to determine the true benefit of combination therapy\(^4\)\(^7\).

Many CF centres (>90% of European centres) select antibiotics based on the susceptibility profile from the most recent sputum samples\(^4\)\(^8\). Aaron et al have demonstrated that for the majority of adult patients suffering an infective exacerbation of cystic fibrosis, the infecting organism is that chronically infecting rather than a newly acquired organism\(^4\)\(^9\). Of note, this has been shown to note be the case for children with CF\(^5\)\(^0\). As such, in adult patients, one would expect a similar antibiotic susceptibility profile between samples if no new antibiotic treatment had been given in the intervening period. However a recent study has highlighted significant inconsistencies in antibiotic susceptibility profiles taken from a single sputum sample\(^5\)\(^1\). Foweraker et al describe how PA susceptibility profiles from a single sputum sample tested in duplicate in 8 separate laboratories had a very poor correlation. They demonstrated that even when colonies on the culture plate had the same morphotypes (i.e. looked the same) they actually had different susceptibility profiles. The authors suggested that
any process of choosing a single sample of a morphotype for susceptibility testing would not
be predictive for the whole sputum sample and not indicative of the microbial response to
treatment *in vivo*, casting doubt over the true benefit of conventional antibiotic susceptibility
testing and its use in selecting appropriate intravenous therapy.

1.5 What Causes an Acute Pulmonary Exacerbation in CF?

Despite acute pulmonary exacerbations in CF being common, the mechanisms which trigger
them are unknown. In chronic PA infection, it could be hypothesized that when a patient is
clinically stable, there is a balance between both the number and activity of bacteria in the
lung and the patient’s immune response to the organisms. An acute exacerbation could
therefore be triggered by either an increase in the number of bacteria, a change in activity of
the bacteria (expression of new virulence factors), or an upregulation of the immune system;
the latter could be caused by another pathogen such as a virus. The following discussions will
focus on PA infections which are the most extensively studied in experimental models.

*Bacterial Levels During Acute Exacerbations*

A simple explanation for the cause of an acute exacerbation would be an overgrowth of
bacteria in the lung which triggers an immune response and the clinical symptoms of an
exacerbation. Previous studies provide conflicting data. Ordoñez et al have previously shown
significant decreases in PA levels from induced sputum samples during treatment of
exacerbations. A recent study examined the sputum density of PA (and total bacterial
density) in the weeks leading up to an exacerbation using a quantitative PCR method. Using
this technique it was shown that there were no changes in either total bacterial density
or PA density prior to the onset of symptoms of an acute exacerbation. Equally a study has
shown non-significant changes in bacterial density during treatment of an exacerbation, and
others have reported no relationship between lung function improvement and changes in PA
density\textsuperscript{39,55}. It would seem therefore that this simple explanation of changes in bacterial numbers causing acute exacerbations may not completely fit with the experimental data.

\textit{Effect of Viral Infections on Acute Exacerbations}

Viral infections have been shown to be relatively common during acute pulmonary exacerbations of CF. For example, in one paediatric study viral particles were detected in 40\% of patients experiencing an exacerbation, compared with only 9\% of those who were clinically stable\textsuperscript{56}. It has also been shown that new bacterial colonisation is more common during seasons when viral infections are more common, with one study showing that 85\% of new PA infections followed within 3 weeks of a viral upper-respiratory tract infection\textsuperscript{57}. Viral infections have been shown to denude respiratory epithelium which may make bacterial adhesion easier. Equally, rhinovirus has been shown to disrupt the PA biofilm allowing motile bacteria to escape which may trigger an exacerbation\textsuperscript{58}.

\textit{Change in P.aeruginosa Activity}

PA has a number of virulence factors that can be activated to facilitate chronic infection. Not all bacteria have all virulence factors active at any one time, many of them activated through genetic mutation. Activation of some of these virulence factors may lead to an acute exacerbation.

The remainder of this discussion will examine PA more closely and some of the virulence factors that may be implicated in an acute exacerbations and how some of these factors have been studied.

\textit{1.6 Pseudomonas aeruginosa}

\textit{Pseudomonas aeruginosa} is a gram negative organism that is ubiquitous in the environment. It has been detected in a diverse range of habitats from garden soil to jet fuel, but natural
carriage by humans is relatively rare. It is however an opportunistic pathogen and is seen in a number of hospital settings such as ventilator-associated pneumonia in intensive care units, urinary tract infections in patients with urinary catheters and soft tissue infections in burns patients. It is also, as previously mentioned, the commonest respiratory pathogen in adult CF patients.

**Virulence Factors**

How is PA able to survive and grow in such a diverse range of habitats?

It can utilise an armamentarium of virulence factors in both aerobic and anaerobic conditions that allow it to persist and thrive in the CF airway. These include structural feature such as flagella, pili, the lipopolysaccharide coating of the outer membrane and a type III secretion system; as well as the ability to secrete compounds such as proteases, exotoxins and alginate. Bacteria can also form into a structure known as a biofilm - a way of aggregating large numbers of bacteria together and protecting them from external agents. Equally, in common with a number of bacteria, PA utilises an organism-to-organism signalling system, known as quorum sensing which allows communities of bacteria to communicate.

**Biofilms**

Microbial biofilms are populations of micro-organisms that are concentrated at an interface (usually solid-liquid) and are typically surrounded by an extracellular polymeric substance (EPS) matrix.

The transition from a single cell planktonic phenotype of PA to a biofilm is thought to be a critical moment of conversion in the setting of CF lung disease. Initial infection with PA in CF is thought to be with environmental, planktonic PA. At some time point later, conversion to a biofilm mode growth occurs, a mode of growth which may make eradication of the bacteria from the airway more difficult and as such lead to chronic infection.
**In vitro Models of PA Biofilm Formation**

Sauer documented the 5 stages of PA biofilm formation in an *in vitro* model\(^5^9\).

**Stage 0 – Planktonic**

This is the wild-type, motile organism

**Stage I – Reversible attachment**

The PA cell attaches transiently to the surface via the cell-pole. This has been shown to be mediated by PA flagella and pili; mutants not exhibiting flagella or pili have much lower rates of surface attachment\(^6^0\).

**Stage II – Irreversible attachment**

PA bacteria attach permanently to the surface by re-orientating to longitudinal cell axis. At this point the bacteria become non-motile, and begin to cluster. Sauer noted activation of the *las* quorum-sensing system during this period of development. Both mutants with a defect in the *las* quorum sensing system and wildtypes produce EPS. However, *las* mutants do not exhibit an EPS matrix where bacterial cells lie spaced apart with EPS between them, a structure thought to be important for biofilm production.

**Stage III – Maturation-1**

During this stage cells are seen to layer into clusters with layer thickness >10μm and protein expression by the cell is massively upregulated. Among the proteins upregulated are Arc proteins, which are thought responsible for the ability of the bacteria to undertake anaerobic processes\(^6^1\), suggesting that cells in the biofilm are exposed to an oxygen depleted environment.
Stage IV – Maturation-2

At this stage the cell cluster layer reaches its maximum thickness of up to 100μm and the bacteria are at their most phenotypically different from planktonic bacteria, with greater than 50% of detectable proteins undergoing changes in regulation. Equally there is a large difference in protein expression from the Maturation-1 stage, possibly related to the high numbers of cells experiencing an anaerobic or low oxygen environment. Cell clusters are seen to be detached from the surface during this stage.

Stage V – Dispersion

In this final stage of development some bacteria develop the ability to become motile and swim away from the interior of cell cluster through pores and channels that develop, presumably to obtain access to better nutrients outside of the cluster. At this point a number of proteins are downregulated and the bacteria more closely resemble the planktonic bacteria compared with Maturation-2 stage bacteria.

*In vivo PA Biofilms*

Can Sauer’s model of biofilm formation be directly extrapolated to the complex environment of the CF airway? Work by Worlitzsch et al in a chronic infection model suggests that rather than occurring at the epithelial cell surface, biofilm formation takes place in the intraluminal space. They demonstrated that, using both electron microscopy and immuno-localisation techniques of explanted CF lungs, PA bacteria did not attach to the airway epithelial cells. Over 95% of organisms were localised to the airway lumen 5-17μm distant from the cell surface in spherical colonies and the rest were positioned between 2-5μm from the cell surface. They also demonstrated preferential binding of PA bacteria to mucus rather than airway epithelial cells. The spherical colonies of bacteria seen in the intra-luminal space were
shown to be oxygen depleted (which is in concordance with Sauer’s in vitro studies showing upregulation of proteins involved in anaerobic respiration) and PA was demonstrated to respond to hypoxia by increasing alginate production, a major component of the EPS matrix of the biofilm. They determined that, in CF, there was a hypoxic gradient in the mucus adherent to the airway surface caused by consumption of oxygen by the hyperactivity of the ENaC channel. They postulate that this gradient encourages PA to produce alginate and develop a biofilm.

If, in chronic infections, PA bacteria are not infecting the airway cells, how can we account for the intense inflammation and neutrophilic infiltration seen in the CF airway? The answer may lie in the response of the innate immune system to the biofilm or possibly quorum sensing molecules.

**Pseudomonas aeruginosa and the Innate Immune System**

As discussed earlier, chronic infection with PA is usually associated with mutation of the planktonic PA to a mucoid phenotype. This mutant produces alginate which acts as a protective barrier for the bacteria against the immune response. A number of studies have identified that PA bacteria with alginate production are associated with persistent infection in murine models\(^63;64\). High levels of alginate produced by PA have also been implicated in the ability of the bacteria to evade the complement system\(^65\).

The development of the PA biofilm is also an important step in protecting the bacteria from the immune system. Experimental biofilms have been shown to be associated with an accumulation of neutrophils close to the biofilm\(^66\). Within the biofilm, PA bacteria produce rhamnolipids (among other virulence factors). These compounds have been shown to be active against neutrophils, causing cell lysis\(^67\). While the full response of the immune system to chronic biofilm infection is still being examined, a theme of the research is the innate
immune response to the bacteria may well be contributing to the disease process with large quantities of inflammatory cells unsuccessfully attacking the biofilm and releasing toxic substances into the airway. The ability of the bacteria to lyse neutrophils leads to large quantities of inflammatory mediators being released into the airway.

As mentioned the PA bacteria in a biofilm have a number of virulence factors capable of protecting the bacteria from the immune system, with many of these virulence factors being controlled through quorum-sensing signalling systems.

**1.7 Quorum Sensing Systems in *Pseudomonas aeruginosa***

Quorum sensing (QS) systems are mechanisms by which cell-to-cell communication can occur in bacterial populations. Each bacterium secretes a small amount of QS molecules. As the number of bacteria increases, the level of QS molecules increases in the extracellular environment and diffuses through the cell membrane of nearby bacteria. At a threshold intracellular concentration of these molecules, target QS genes are activated within the bacterium which may alter the expression of virulence factors. It therefore acts, as the name suggests, as a density-dependent regulatory system.

PA has a complex multi-compound QS system. The bulk of the QS system is controlled by two genes, the *lasR* and *rhlR* genes, which regulate over 10% of the entire PA genome. The two principal QS proteins that bind to and activate these genes are *N-* (3-oxododecanoyl) homoserine lactone (3-oxo-C₁₂-AHL), which binds to the *lasR* gene, and *N*-butanoylhomoserine lactone (C₄-AHL), which binds to the *rhlR* gene. These proteins are from a group known as acylated homoserine lactones (AHLs). More recently a further group of molecules, known as 2-alkyl-4(1H)-quinolones (AQ), have been shown to be important in PA QS systems.
QS-controlled genes encode a number of virulence factors required by PA to survive in the CF airway. The presence of 3-oxo-C12-AHL has been shown to be required for PA biofilm formation\(^{70}\) and it was through measuring of QS proteins in CF sputum that it was initially determined PA grew in a biofilm in CF airway disease.\(^{71}\)

QS-systems are also involved the development of resistance to some antibiotics. Suppressors of the PA QS system have been shown to increase susceptibility to tobramycin\(^{72}\), and mutations leading to deletion of QS genes have been shown to increase PA susceptibility to oxidative stress and make them more readily phagocytosed by polymorphonuclear leukocytes\(^{73}\).

The QS molecules may also play an important role in the relationship between PA and the CF airway. As previously mentioned the PA biofilm is located within intraluminal mucus rather than attached to the epithelial cells in the CF lung. 3-oxo-C12-AHL in particular has been shown to stimulate human airway epithelial cells to produce IL-8, a potent chemo-attractant for neutrophils, induce cyclo-oxygenase-2 (Cox-2), a pro-inflammatory enzyme, in human lung fibroblasts and stimulate the production of PGE\(_2\) in fibroblasts\(^{74,75}\). PGE\(_2\) is known to induce mucus secretion, vasodilatation and oedema within the airway.

Therefore it may be that the QS molecules are activating inflammatory pathways in the CF airway which may augment the upregulated inflammatory pathways previously discussed. No work has yet been done to examine QS molecules during infective exacerbations of CF due to PA, which will be a further area of study in this thesis.

It has been noted by some investigators that QS signalling systems are lost in chronic PA infections through genetic mutation\(^{76}\). However it may be that at times of acute exacerbation, QS-proficient organisms proliferate and play a role in the pathogenesis of an infection. Certainly it would appear that QS-proficient organisms appear more able to cause acute
infections. A study of mechanically ventilated patients in an intensive care setting identified that patients infected with QS-proficient PA were more likely to develop ventilator-associated pneumonia than those without.77

QS-systems may also allow interaction between different species of bacteria. In the condition otitis media, co-infection with *H.influenza* and *M. cattarhalis* promotes increased resistance to antibiotics. The QS molecule autoinducer-2, secreted by *H.influenza* was identified as a promoter of *M.cattarhalis* biofilm production and increased resistance to antibiotic therapies78.

A number of compounds have been shown to disrupt QS signalling. One such compound is the macrolide antibiotic, azithromycin. In chronic PA infections the long term use of azithromycin is associated with reduced frequency of pulmonary exacerbations and improved lung function79. While the full mechanism of this action has yet to be elucidated a number of studies have clearly shown a decrease in QS molecule production by PA in the presence of azithromycin in urinary tract infections, ventilator-associated pneumonia and a CF mouse model80-82.

1.8 Hypermutator Strains of *Pseudomonas aeruginosa*

The wide range of virulence factors seen in PA is not all expressed at once. For example PA bacteria causing an acute ventilator-associated pneumonia is phenotypically very different from that seen in chronic CF lung infection. Changing virulence factors are acquired through mutations which occur as an adaptation to their environment.

Evolutionary genetics would suggest that, in general, the spontaneous mutation rate of bacteria should be low83. The majority of genetic mutations involve DNA deletion or errors of transcription which lead to the loss of cellular function and cell death. Beneficial mutations to the bacteria are theoretically relatively rare. However in reality that is not the case; PA is seen
to mutate regularly in CF lungs to improve the fitness of the bacteria. One study examined the
genetic mutations of PA in a single patient over an 8 year period commencing at the time of
initial infection. They noted 68 mutations over the time period including the loss of a number
of genes coding for virulence factors required for initial infection such as twitch motility and
serotype-specific antigenicity.\textsuperscript{84}

Bacteria with high mutation rates were first identified in experimental colonies of \textit{E. Coli}.\textsuperscript{85} These “hypermutators” have a mutation rate up to a thousand times greater than wild-type
caused by a defect in their DNA repair system. While the majority of these mutations will be
deleterious, occasionally, and at a much higher frequency than wild-type bacteria, a beneficial
mutation will occur allowing a new virulence factor to be expressed. PA hypermutators have
also been identified and shown to exist in high numbers in CF sputum samples.\textsuperscript{86}

While PA hypermutators have been linked to the development of antibiotic resistance and
development of a mucoid phenotype,\textsuperscript{87,88} it is likely that the ability to hypermutate is also
associated with a biological cost. During \textit{in vitro} competition tests, wild-type outcompeted
hypermutator strains and \textit{in vivo} experiments, using a murine model of chronic infection,
demonstrated hypermutators were less able to establish infection.\textsuperscript{89} These studies suggest that
at times of changing airway environment e.g. acute infective exacerbations, hypermutation
helps bacterial communities adapt to changes in the local environment. In a stable bacterial
community however they may be less useful because of their associated biological cost and
decreased virulence. As such, hypermutators levels may drop after treatment of acute
infections.
1.9 Study Hypotheses

This study examined three major hypotheses:

**Study Hypothesis 1**

“*P. aeruginosa* hypermutators are representative of a bacterial population under stress rather than acting as a more pathogenic organism. Their presence does not relate to clinical outcome but may be associated with increased antibiotic resistance emerging”

**Study Hypothesis 2**

“Increased microbiological diversity represents a balanced community of bacteria. The presence of a diverse population therefore predicts a better outcome for treatment than when a population consists of a small number of virulent organisms predominate”

**Study Hypothesis 3**

“Quorum sensing molecules released by *P.aeruginosa* contribute to upregulation of inflammatory pathways within the CF airway. Increasing populations of QS-producing organisms trigger acute exacerbations and treating this population will resolve acute exacerbations. Persistence of QS production would therefore reflect non-resolution of acute infection”

1.10 Plan of Investigation

The thesis examined the treatment of an infective exacerbation of CF using intravenous antibiotic therapy as its study model. The study recruited patients prior to commencing antibiotic therapy and then followed them through treatment and to a stable point following completion of therapy.
Aims of the Thesis

- A clinical tracking study will be performed to determine the clinical factors associated with treatment response to infection and predictors of successful treatment.

- A microbiological study using culture-based microbiology will identify the effect of *P. aeruginosa* morphotypes and antibiotic resistance patterns on treatment response, the effect of antibiotic therapy on bacterial numbers and the effect of other bacteria on treatment response.

- The presence of *P. aeruginosa* hypermutators using a culture based assay will be determined. This will be correlated with clinical outcomes such as improvement in lung function, changes in inflammatory markers, and the development of antibiotic resistance.

- Using 16s rRNA gene pyrosequencing from collected sputum samples, bacterial populations will be examined at a molecular level to determine relationships between bacterial diversity and treatment outcomes.

- Quorum sensing molecule concentrations will be measured during treatment to determine whether a relationship exists between bacterial signalling and clinical measures, as well as treatment response.
CHAPTER 2

THE CF INFECTIVE EXACERBATION – A CLINICAL TRACKING STUDY

2.1 Introduction
The CF exacerbation is a well documented but perhaps poorly understood clinical occurrence. As part of this study into bacterial ecology in CF exacerbations it is first important to examine the exacerbations at a clinical level, both with objective markers such as lung spirometry and inflammatory markers, but also subjective markers as reported by quality of life questionnaires completed by the patients.

While the main purpose of the clinical tracking study is to obtain clinical data and matched sputum samples for a study of the microbiology of CF exacerbations, it also provides an opportunity for a detailed investigation of the CF exacerbation in terms of the response of lung function and inflammatory markers to treatment and to determine if any factors measured at the start of treatment appear to predict treatment outcome. In CF clinical care the aim of treating exacerbations is to preserve lung function and this study therefore examined whether patients returned to their baseline lung function (a treatment success) or completed treatment without returning to baseline (a treatment failure).

2.2 Methods

Ethical Approval and Study Registration

Ethical approval for the study was sought from the Central London Research Ethics Committee and given a favourable opinion (REC reference 11/H0713/7). The trial was also registered on clinicaltrials.gov (Identifier: NCT01306279).
Study Design

The study was designed as a longitudinal study, observing patients during the treatment of an infective exacerbation of CF (IECF) and at a stable time after their infection has been treated. Sputum samples collected in this tracking study were processed as discussed below and used in further assays along with the clinical measurements collected as described in later chapters.

Participant selection and recruitment

Patients were recruited from the adult cystic fibrosis patient group attending the Royal Brompton Hospital (RBH). Only those patients who were chronically infected with *Pseudomonas aeruginosa* were included. Chronic infection was defined as a minimum of 2 positive sputum cultures in the preceding 6 months prior to study recruitment. This was a local modification of the Copenhagen criteria\(^9\), requiring persistent presence of PA over a 6 month period (most patients have sputum samples taken every 3 months).

Patients were invited to participate in the study when they attended RBH to commence intravenous antibiotic treatment for an IECF.

Inclusion and Exclusion Criteria

Patients were included in the study only if they met the criteria for a definition of an IECF as set out by the 1994 CF Foundation Microbiology and Infectious Disease Consensus Conference as described on page 27-28. These criteria were decided upon as they were based on clinical consensus rather than designed for a particular study.

Patients were excluded from the study if any of the following applied:

1. Age under 16 years at time of recruitment.
2. Unable to give consent or patients with significant mental health problems.
3. Co-existent active allergic bronchopulmonary aspergillosis (ABPA) requiring a change in steroid or anti-fungal therapy.

4. A previous participant in the study.

5. Antibiotic therapy for an exacerbation in the previous 4 weeks

**Treatment of Pulmonary Exacerbations**

Study participants received a combination of two antibiotics, generally a beta-lactam antibiotic and an aminoglycoside, as per routine clinical practice at RBH. Antibiotic choice was determined by the treating clinician based on previous antibiotic sensitivity patterns and known drug allergies. Maintenance oral or inhaled antibiotics, oral corticosteroids, and inhaled dornase alpha were continued if they had been on these therapies at a stable dose for the preceding 2 months.

Subjects were scheduled to receive a 14 day course of intravenous antibiotics as was routine clinical practice at RBH at the time of the study.

Subjects could receive the antibiotic therapy either in the hospital or at home. Patients who received their antibiotic therapy at home were asked to return to RBH on the study days for assessment.

**Study Visits**

Subjects were assessed on days 1, 7, and the last day of treatment (day 10 or 14) of their antibiotic therapy. The day 1 assessments were completed prior to commencing antibiotic therapy. Data was also collected from the subject’s medical records of the best spirometry values in the preceding six months.
Study Questionnaires
At each study visit, subjects were asked to complete 2 quality of life (QOL) questionnaires, the Brompton CF Questionnaire (BCFQ) and the Cystic Fibrosis Questionnaire-revised (CFQR).

The CFQR is a well recognised and validated QOL questionnaire. In this study the Adolescent and Adult UK version was used. The form (see appendix) is 50 questions pertaining to all areas of symptoms related to CF. The resultant score is given in 13 separate domains. In this study only the respiratory domain score was analysed. A maximum score is 100, the minimum being 0, and a lower score is indicative of a greater burden of symptoms.

The BCFQ is a shorter questionnaire of 38 questions providing a single score out of 100. In this questionnaire a higher score is indicative of increased symptoms.

Spirometry
Subjects performed spirometry using forced manoeuvres to obtain FEV\textsubscript{1}, FVC and FEF\textsubscript{25-75}. The best of a maximum of 3 attempts made on a hand held spirometer (Easyone™ Spirometer, ndd Medizintechnik, Switzerland) was recorded, as per clinical practice at RBH. Percent predicted values were calculated using the European Coal and Steel Community (ECSC) reference values.

Blood Testing
A sample of blood (approximately 5ml) was obtained from patients at each study day during antibiotic therapy. Samples were processed in the haematology and biochemistry labs at RBH for measures of total white cell count, neutrophil count and C-reactive protein (CRP).

Sputum Sample
A sputum sample was collected from each subject and processed as described in the following section. Patients who were non-productive remained in the study.
Sputum Processing

Sputum was collected from patients in sterile pots and processed within 30 minutes of expectoration with aliquotting into 4 samples under sterile conditions.

A – Routine microbiology culture and sensitivities
B – *Pseudomonas* hypermutator assay
C – Identification of bacteria using 16SRNA identification techniques
D – Quorum sensing molecule assay

A minimum of 0.5ml of sputum was aliquoted into each pot.

Sample A was transferred to the microbiology laboratory at RBH for further processing as per usual clinical practice. Sample B was shipped at ambient temperature overnight to a separate facility (Papworth Hospital) for the hypermutator assay described in Chapter 4. Samples C and D were immediately placed on dry ice and then stored as whole sputum at -80°C for later testing.

Treatment Outcomes

For the purpose of analysis a successful outcome of antibiotic treatment was defined as a return of FEV₁% predicted to within 10% of their previous stable value (defined as the best FEV₁ in the preceding 6 months) and a CRP less than 30 at the completion of antibiotic therapy. This spirometry definition was based on a large registry data study that defined failure to recover baseline lung function as being less than 90% of baseline⁹¹, with CRP value being based on clinical experience.

Subject Monitoring after Completion of Antibiotic Therapy

Patients were followed up as per routine clinical practice. One month after completion of antibiotic therapy patients were reviewed in outpatient clinic and measures of spirometry were recorded along with collection of a sputum sample which was processed as above. Patients were then monitored for up to 6 months after treatment while under usual clinical
follow-up for any further exacerbations to determine time to next exacerbation (which could be treated with either oral or intravenous antibiotics).

**Statistical Methodology**

Continuous variables were compared using Mann-Whitney testing for non-paired data, and Wilcoxon Matched pairs test for paired data. Categorical variables were examined using Fisher’s exact test.

Correlations were performed using Spearman’s rank correlation co-efficient. Time to next exacerbation data was examined using a log rank test.

All data is expressed as median values with the interquartile range. All statistical analysis was performed using Graphpad Prism (GraphPad Software Inc).

No power calculations were performed for this study as it was intended as a hypothesis generating pilot study to guide future larger studies. Equally no correction has been made for multiple comparisons to assist in generation of hypotheses, rather than confirmation of an effect.
### 2.3 Results

**Patient Population**

Subjects were recruited from 01/06/2010 until 30/09/2011. Over this time, 30 subjects consented to participate in the study. Two patients were withdrawn from the study in the first week (1 withdrew consent, 1 began treatment for allergic bronchopulmonary aspergillosis), one patient did not culture PA from sputum samples. Data from 27 subjects were analysed.

The median age of subjects was 30 years, and there were 12 males and 15 females. Thirteen of the subjects (48%) were homozygous for the phe508del mutation, ten were heterozygote. Only one patient did not carry the phe508del mutation and genotyping was not available for a further 3 patients. Twenty-six patients were pancreatic insufficient. Eight patients had co-existent CF related diabetes. Prior to the exacerbation, subjects had a stable baseline median FEV\textsubscript{1} of 1.6 litres (IQR 1, 2.2), with a median FEV\textsubscript{1} % predicted of 48% (IQR 35, 67).

Baseline characteristics were analysed by comparing treatment outcome groups. Twenty patients were deemed a treatment success and 7 were a treatment failure. When comparing groups by outcomes, those in the treatment success group were significantly older than those in the treatment failure group (median age 37 (28, 46) vs. 23 (18, 30), *p*=0.04). There were no differences between the treatment outcome groups when comparing gender, phe508del homozygosity, pancreatic insufficiency or the presence of diabetes.

There were no significant differences in stable lung function prior to the exacerbation between the treatment success and failure groups. However, there was a significantly longer time from stable measure to exacerbation in the treatment failure group as compared with the treatment success group as demonstrated in table 2.1. There were no differences between the groups when comparing regular medications.
Table 2.1 Baseline characteristics of study subjects. Last stable lung function was the best measure in the preceding 6 months. Data is presented as all subjects and then a comparison of groups defined by treatment outcomes. p values are determined with Mann-Whitney test for continuous variables and Fishers exact test for categorical variables.

<table>
<thead>
<tr>
<th></th>
<th>All (n=27)</th>
<th>Treatment Success (n=20)</th>
<th>Treatment Failure (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Median (IQR))</td>
<td>30 (23,43)</td>
<td>37 (28,46)</td>
<td>23 (18,30)</td>
<td>0.04</td>
</tr>
<tr>
<td>M/F</td>
<td>12:15</td>
<td>10:10</td>
<td>2:5</td>
<td>0.41</td>
</tr>
<tr>
<td>phe508del homozygous</td>
<td>13</td>
<td>8</td>
<td>5</td>
<td>0.21</td>
</tr>
<tr>
<td>Pancreatic insufficiency</td>
<td>26</td>
<td>19</td>
<td>7</td>
<td>1.0</td>
</tr>
<tr>
<td>CF related diabetes</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Last Stable Lung Function- median (IQR)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁ (litres)</td>
<td>1.6 (1.0,2.2)</td>
<td>1.6 (1.0,1.9)</td>
<td>2.1 (1.0,2.5)</td>
<td>0.52</td>
</tr>
<tr>
<td>FEV₁ %pred</td>
<td>48 (35,67)</td>
<td>46 (35,62)</td>
<td>51 (33,78)</td>
<td>0.42</td>
</tr>
<tr>
<td>FVC (litres)</td>
<td>2.8 (2.0,3.7)</td>
<td>2.8 (2.1,3.8)</td>
<td>2.8 (2.0,3.1)</td>
<td>0.30</td>
</tr>
<tr>
<td>FVC %pred</td>
<td>78 (66,92)</td>
<td>81 (67,93)</td>
<td>71 (55,91)</td>
<td>0.26</td>
</tr>
<tr>
<td>Median days from stable measure to exacerbation</td>
<td>149 (94,207)</td>
<td>130 (82,170)</td>
<td>240 (160,260)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Regular CF medications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhaled Colomycin</td>
<td>21</td>
<td>15</td>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>Inhaled Tobramycin</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>Inhaled DNase</td>
<td>24</td>
<td>17</td>
<td>7</td>
<td>0.55</td>
</tr>
<tr>
<td>Inhaled Hypertonic Saline</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0.63</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>23</td>
<td>16</td>
<td>7</td>
<td>0.35</td>
</tr>
<tr>
<td>Flucloxacillin</td>
<td>11</td>
<td>8</td>
<td>3</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Antibiotic Therapy

Duration of Antibiotic Therapy

Twenty seven subjects completed courses of intravenous antibiotics. Nineteen subjects completed a 14 day course of antibiotics with the remaining 8 completing a 10 day course of antibiotics. Patients who stopped treatment at 10 days did so due to patient preference with clinician approval. There was no relationship between antibiotic duration and treatment success, day 1 lung function or lung function improvement. Day 1 FEV₁ as percentage of baseline values were not significantly different between those who received 10 days of treatment (71% (59,96)) compared with those who received 14 day (87% (76,90)), p = 0.42. Median percentage improvement in FEV₁ for 10 days therapy was 19% compared with 16% for 14 days therapy (p>0.05). Median FEV₁ as a percentage of baseline values at the end of treatment for those stopping at day 10 were 92% (87,100) with a minimum value of 65%.

Antibiotic Choice

As per local clinical guidelines, all subjects received two intravenous antibiotics, a beta-lactam antibiotic and an aminoglycoside or colomycin. Twelve subjects received ceftazidime, 13 received meropenem and 2 received aztreonam. Of the aminoglycosides/colomycin, 22 received tobramycin, 4 received colomycin and 1 subject received gentamicin.

There were no significant differences in treatment success or improvement in FEV₁ when comparing antibiotic groups. Median percentage improvement in FEV₁ in those receiving ceftazidime was 19% compared with 18% for those receiving meropenem (p=0.93) as shown in Figure 2.1. The number of patients receiving aztreonam was too small for non-parametric comparison.
Figure 2.1 The % change in FEV$_1$ during treatment of an exacerbation with a comparison of subjects receiving ceftazidime with subject receiving meropenem. Data is presented as a box-and-whisker plot with the box representing median value with interquartile range and the whiskers representing minimum and maximum values. No significant difference is seen between groups (p=0.93)
Figure 2.2  The % change in FEV₁ during treatment of an exacerbation with a comparison of subjects receiving 10 days of treatment with those receiving 14 days. Data is presented as a box-and-whisker plot. The boxes represent median values and interquartile ranges. The whiskers represent maximum and minimum values. No significant difference is seen between groups (p= 0.42)

**Antibiotic Usage and Time-to-Next-Exacerbation (TTNE)**

Time to next exacerbation was not affected by either antibiotic choice or antibiotic duration. When comparing beta-lactam antibiotics, the median TTNE following a course of meropenem was 66 (33,157) days compared with 92 (72,157) for those subjects who received ceftazidime (p=0.78).

Subjects who received ten days of antibiotic therapy had a median TTNE of 96 days, compared with 73 days for those who received 14 day courses, (p=0.83).
Figure 2.3  A comparison of time to next exacerbation values in subjects receiving meropenem or ceftazidime. Data is presented as a Kaplan-Meier plot, with the x-axis representing time to next exacerbation. No significant difference is seen between groups (p=0.78)

Figure 2.4  A comparison of time to next exacerbation values in subjects receiving 10 or 14 days of antibiotic therapy. Data is presented as a Kaplan-Meier plot, with the x-axis representing time to next exacerbation. No significant difference is seen between groups (p=0.83)
Lung Function Changes During the Exacerbation Treatment

FEV<sub>1</sub>

Examining the subject group as a whole there was a significant drop in median FEV<sub>1</sub> at the start of the exacerbation (1.3L (0.9,1.5) as compared with the stable, pre-exacerbation baseline value (1.6L (1.0,2.2)), p= <0.0001. There was a significant rise in median FEV<sub>1</sub> during treatment which was seen at day 7 and maintained throughout the treatment to the stable post-treatment result (Table 2.2 and Figure 2.5a).

When examining subgroups based on treatment success or failure, both groups had experienced a significant decrease in median FEV<sub>1</sub> from baseline to the start of the exacerbation as documented in table 2.2. In the treatment success group a significant rise in median FEV<sub>1</sub> was seen at day 7 and maintained through treatment and to the stable post-treatment measure. In the treatment failure group there was no significant difference between the day 1 and day 7 median FEV<sub>1</sub>. However, by the end of treatment a significant rise in FEV<sub>1</sub> was noted but this was not continued onto the stable post-treatment value. There were no significant differences between the groups for median FEV<sub>1</sub> values at any time-point of the study (Table 2.2 and Figures 2.6 and 2.9a).

FEV<sub>1</sub> %predicted

As with FEV<sub>1</sub>, when looking at the subject group as a whole, there was a significant drop in median FEV<sub>1</sub> % predicted from stable baseline to the start of the exacerbation. A significant improvement was seen at day 7 and this improvement was maintained through all measured time-points including the stable post-treatment value (Table 2.2 and Figure 2.5b).

When examining subgroups, the significant drop in FEV<sub>1</sub> % predicted was seen at the start of exacerbations in both treatment success and failure groups with no significant difference in
median values between the groups. In the treatment success group there is a significant improvement in FEV\(_1\) % predicted at day 7 which is maintained throughout all following time points. In the treatment failure group no significant improvement in FEV\(_1\) % predicted is seen at any treatment time point. (Table 2.2 and Figures 2.7 and 2.9b).

**FEV\(_1\) as a percentage of the baseline value (FEV\(_1\) %OBL)**

In the study population as a whole there was a significant drop in FEV\(_1\) %OBL from a baseline value of 100% to the day 1 value. After the commencement of treatment there were significant improvements in FEV\(_1\) %OBL for all time-points (Table 2.2 and Figure 2.5c).

When comparing subgroups divided by treatment success or failure, there was a significant drop in median FEV\(_1\) %OBL at day 1 in both groups, but the value in the treatment failure group was significantly lower (65% vs. 89%, \(p=0.001\)). In the treatment success group there were significant improvements in median FEV\(_1\) %OBL at all subsequent time groups. In the treatment failure group the improvement in median FEV\(_1\) %OBL was only seen at the end of treatment values. The median value of FEV\(_1\) %OBL was significantly greater in all treatment time-points for the treatment success group with the exception of the stable post-treatment value (Table 2.2 and Figures 2.8 and 2.9c) as would be expected given the definition of treatment success was based on the FEV\(_1\) %OBL value at the end of treatment being greater than 90%.

**FVC and FVC% predicted**

Overall there was a significant drop in median FVC from baseline values to the start of an exacerbation. After commencing antibiotic therapy there were significant improvements in median FVC for all time-points. In both treatment success and treatment failure subgroups
there were significant drops in median FVC in both groups with a significant improvement seen at the end of treatment. A similar pattern was seen with FVC % predicted.

**$FEF_{25.75} \%$ predicted**

When reviewing all patients the median $FEF_{25.75} \%$ predicted values improved marginally at day 7 and day 10 but this significance in improvement was lost by the end of treatment values. A similar pattern was seen in the treatment success group but no significant changes in $FEF_{25.75} \%$ predicted were seen in the treatment failure group.

<table>
<thead>
<tr>
<th></th>
<th>All (n=27)</th>
<th>Treatment Success (n=20)</th>
<th>Treatment Failure (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>$FEV_1$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.6 (1.0,2.2)</td>
<td>1.6 (0.9,1.9)</td>
<td>2.1 (1.0,2.5)</td>
<td>0.35</td>
</tr>
<tr>
<td>Day 1</td>
<td>1.3 (0.9,1.5)*</td>
<td>1.3 (0.9,1.6)*</td>
<td>1.3 (0.7,1.4)*</td>
<td>0.65</td>
</tr>
<tr>
<td>Day 7</td>
<td>1.7 (1.1,2.0)†</td>
<td>1.7 (1.4,2.0)†</td>
<td>1.3 (0.9,1.9)</td>
<td>0.56</td>
</tr>
<tr>
<td>End of Treatment</td>
<td>1.6 (0.9,2.1)†</td>
<td>1.6 (1.0,2.2)†</td>
<td>1.5 (0.9,2.2)†</td>
<td>0.79</td>
</tr>
<tr>
<td>Stable Post</td>
<td>1.4 (0.8,1.9)†</td>
<td>1.5 (1.2,2.2)†</td>
<td>0.8 (0.7,1.6)</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>$FEV_1 %$ predicted</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>48 (35,67)</td>
<td>46 (35,62)</td>
<td>51 (33,78)</td>
<td>0.42</td>
</tr>
<tr>
<td>Day 1</td>
<td>39 (29,48)*</td>
<td>41 (31,48)*</td>
<td>33 (24,48)*</td>
<td>0.30</td>
</tr>
<tr>
<td>Day 7</td>
<td>51 (36,60)†</td>
<td>52 (40,65)†</td>
<td>37 (25,56)*</td>
<td>0.16</td>
</tr>
<tr>
<td>End of Treatment</td>
<td>48 (27,59)†</td>
<td>51 (37,59)†</td>
<td>44 (25,67)</td>
<td>0.84</td>
</tr>
<tr>
<td>Stable Post</td>
<td>44 (26,58)†</td>
<td>47 (32,67)†</td>
<td>24 (24,45)</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>$FEV_1$ as % of baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>85 (69,93)*</td>
<td>89 (81,95)*</td>
<td>65 (57,71)*</td>
<td>0.001</td>
</tr>
<tr>
<td>Day 7</td>
<td>101 (89,105)†</td>
<td>104 (96,115)†</td>
<td>83 (73,91)</td>
<td>0.002</td>
</tr>
<tr>
<td>End of Treatment</td>
<td>102 (89,106)†</td>
<td>104 (96,111)†</td>
<td>86 (68,87)†</td>
<td>0.0001</td>
</tr>
<tr>
<td>Stable Post</td>
<td>97 (89,105)†</td>
<td>97 (90,105)†</td>
<td>75 (73,99)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 2.2 Spirometry values during treatment CF exacerbation for all patients and then a comparison of groups defined by treatment outcome. Baseline values is the best value in the preceding 6 months. * p<0.05 compared with baseline value. † p<0.05 compared with day 1 value.
Comparing changes in lung function between treatment outcome groups

The change in FEV₁, FEV₁ % predicted and FEV₁ as a percentage of baseline were examined between baseline values and day 1 values, and between day 1 values and the end of treatment. When comparing the change from baseline to the start of the exacerbation there was a significantly greater decrease in all of the measured parameters for the treatment failure group as compared with the treatment success group. No significant differences were seen between treatment success and treatment failure groups when comparing changes in spirometry values between day 1 of treatment and the end of treatment (Table 2.3)

<table>
<thead>
<tr>
<th>Change in FEV₁</th>
<th>All (n=27)</th>
<th>Treatment Success (n=20)</th>
<th>Treatment Failure (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline to day 1</td>
<td>-0.3 (-0.7,-0.1)</td>
<td>-0.2 (-0.4,-0.1)</td>
<td>-0.8 (-1.1,-0.3)</td>
<td>0.01</td>
</tr>
<tr>
<td>Day 1 to End of Rx</td>
<td>0.2 (0.1,0.6)</td>
<td>0.3 (0.1,0.6)</td>
<td>0.2 (0.08)</td>
<td>0.91</td>
</tr>
<tr>
<td>Day 1 to Day 7</td>
<td>0.2 (0.1,0.5)</td>
<td>0.2 (0.05)</td>
<td>0.3 (0.1,0.5)</td>
<td>0.67</td>
</tr>
<tr>
<td>Day 7 to End of Rx</td>
<td>0 (0,0.2)</td>
<td>0.1 (0.2)</td>
<td>0 (-0.1,0.1)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Change in FEV₁ % predicted</th>
<th>Baseline to day 1</th>
<th>Day 1 to End of Rx</th>
<th>Day 1 to Day 7</th>
<th>Day 7 to End of Rx</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline to day 1</td>
<td>-9 (-18,-3)</td>
<td>-7 (-11,-2)</td>
<td>-23 (-30,-9)</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>Day 1 to End of Rx</td>
<td>7 (3,15)</td>
<td>6 (3,15)</td>
<td>8 (0.24)</td>
<td>0.88</td>
<td></td>
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<tr>
<td>Day 1 to Day 7</td>
<td>7 (2,13)</td>
<td>7 (1,13)</td>
<td>6 (3,13)</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Day 7 to End of Rx</td>
<td>1 (-2,3)</td>
<td>1 (-3,3)</td>
<td>-1 (-2,4)</td>
<td>0.69</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Change in FEV₁ as % of baseline</th>
<th>Baseline to day 1</th>
<th>Day 1 to End of Rx</th>
<th>Day 1 to Day 7</th>
<th>Day 7 to End of Rx</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline to day 1</td>
<td>-16 (-34,-8)</td>
<td>-12 (-22,-5)</td>
<td>-35 (-43,-29)</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Day 1 to End of Rx</td>
<td>15 (8,27)</td>
<td>15 (9,27)</td>
<td>16 (4,30)</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>Day 1 to Day 7</td>
<td>15 (4,24)</td>
<td>14 (2,29)</td>
<td>18 (11,22)</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Day 7 to End of Rx</td>
<td>2 (-2,7)</td>
<td>3 (-2,10)</td>
<td>-2 (-9,3)</td>
<td>0.14</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 Median changes in lung function during exacerbation treatment and comparing groups defined by treatment outcomes. Baseline value is the best value in the preceding 6 months. All values are medians with interquartile ranges.
Figure 2.5 (a) Median FEV$_1$ for all subjects at each time point of treatment. Data is presented as a box and whisker plot with boxes representing median and interquartile ranges and whiskers representing minimum and maximum values.

(b) Median FEV$_1$ % predicted for all subjects at each time point of treatment. Data is presented as a box and whisker plot with boxes representing median and interquartile ranges and whiskers representing minimum and maximum values.

(c) Median FEV$_1$ %OBL all subjects at each time point of treatment. Data is presented as a box and whisker plot with boxes representing median and interquartile ranges and whiskers representing minimum and maximum values.
Figure 2.6 A plot of FEV$_1$ during antibiotic treatment for all subjects at each time point with treatment success subjects in blue and treatment failure subjects in red. Baseline value is the best value in the preceding 6 months
Figure 2.7 A plot of FEV$_1$ % predicted during antibiotic treatment for all subjects at each time point with treatment success subjects in blue and treatment failure subjects in red. Baseline value is the best value in the preceding 6 months.
Figure 2.8 A plot of FEV$_1$ as a % of baseline during antibiotic treatment for all subjects at each time point with treatment success subjects in blue and treatment failure subjects in red. Baseline value is the best value in the preceding 6 months.
Figure 2.9 (a) Median FEV$_1$ values with interquartile ranges (error bars) for each time point of antibiotic treatment. Treatment success subjects in blue, treatment failure subjects in red. No significant differences are seen at any time point between groups.

(b) Median FEV$_1$ % predicted values with interquartile ranges (error bars) for each time point of antibiotic treatment. Treatment success subjects in blue, treatment failure subjects in red. No significant differences are seen at any time point between groups.

(c) Median FEV$_1$ as a percentage of baseline values with interquartile ranges (error bars) for each time point of antibiotic treatment. Treatment success subjects in blue, treatment failure subjects in red. Values are significantly different (p<0.05) for the day 1, day 7 and end of treatment values.
Markers of Inflammation During the Exacerbation

*C-reactive Protein (CRP)*

Thirteen patients had CRP levels above the normal range (0-10mg/L) at the beginning of the exacerbation. When examining all patients there was a significant decrease in the median CRP value between the start of antibiotic therapy and day 7 of treatment (10 vs. 4, p<0.002) as demonstrated in Table 2.4 and Figure 2.10. No significant difference was seen in median CRP values between treatment outcome groups; treatment success 9 (5, 24), treatment failure 33 (9, 49)

<table>
<thead>
<tr>
<th></th>
<th>All (n=27)</th>
<th>Rx Success (n=20)</th>
<th>Rx Failure (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRP (mg/L) median and IQR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>10 (6,33)</td>
<td>9 (5,24)</td>
<td>33 (9,49)</td>
<td>0.09</td>
</tr>
<tr>
<td>Day 7</td>
<td>4 (1,9)†</td>
<td>4 (1,8)</td>
<td>9 (4,12)</td>
<td>0.23</td>
</tr>
<tr>
<td>End Rx</td>
<td>4 (1,7)†</td>
<td>2 (1,6)</td>
<td>6 (3,24)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 2.4 CRP median values (with IQRs) during antibiotic therapy and comparison of treatment groups. † p<0.05 compared with day 1 value.
Correlations were performed to examine the relationship between changes in CRP with changes in spirometry values. There were no significant correlations between the change in CRP and absolute change in FEV$_1$ ($r = -0.068$, $p = 0.74$), or between change in CRP and percent change in FEV$_1$ ($r = 0.014$ $p = 0.95$).

**White Cell Count**

The total white cell count reduced significantly in all groups when comparing the start of treatment with day 7 and the end of treatment. There were no significant differences in white cell count between the treatment outcome groups. Analysis of neutrophil counts revealed a similar pattern with significant decreases in neutrophil values in both treatment outcome
groups, but no significant differences between the groups as shown in Table 2.5 and Figures 2.11 and 2.12)

<table>
<thead>
<tr>
<th></th>
<th>All (n=27)</th>
<th>Rx Success (n=20)</th>
<th>Rx Failure (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>White cell count (x10^9/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>13.1 (9.9,14.9)</td>
<td>11.9 (8.7,14.6)</td>
<td>14.3 (12.6,15.7)</td>
<td>0.11</td>
</tr>
<tr>
<td>Day 7</td>
<td>8.8 (6.9,10.8) †</td>
<td>8.7 (6.8,10.8) †</td>
<td>8.9 (7.7,10.9) †</td>
<td>0.69</td>
</tr>
<tr>
<td>End Rx</td>
<td>9.0 (6.5,10.9) †</td>
<td>9.6 (6.7,10.6) †</td>
<td>7.3 (5.5,12.8) †</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Neutrophil count (x10^9/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day 1</td>
<td>9.7 (7.7,12)</td>
<td>8.9 (6.4,12)</td>
<td>11.0 (9.8,14)</td>
<td>0.09</td>
</tr>
<tr>
<td>Day 7</td>
<td>6.2 (4.3,7.5) †</td>
<td>5.5 (4.3,7.4) †</td>
<td>7.0 (4.7,8.9) †</td>
<td>0.40</td>
</tr>
<tr>
<td>End Rx</td>
<td>6.0 (3.9,8.7) †</td>
<td>6.3 (4.2,8.3) †</td>
<td>5.8 (3.1,9.4) †</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Table 2.5 Median total white cell counts and neutrophil counts during exacerbation treatment and a comparison of groups defined by treatment outcome. † p<0.05 compared with day 1 value.
Figure 2.11  Individual total white cell count responses to antibiotic therapy. Treatment success subjects in blue, treatment failure patients in red. No significant differences are seen between outcome groups.
Figure 2.12 Individual neutrophil count responses to antibiotic therapy. Treatment success subjects in blue, treatment failure patients in red. No significant differences are seen between treatment outcome groups.

Relationships between day 1 total white cell and neutrophil counts and the FEV\textsubscript{1} as a percentage of baseline were examined. There were no significant correlations seen. The relationship between changes in lung function and changes in total white cell counts and neutrophil counts was also examined. While in general no significant association was found there was a moderate correlation between the percent change in FEV\textsubscript{1} and change in neutrophil count. Finally the relationship between inflammatory markers at the end of treatment and the time to exacerbation was examined with no significant correlations being found as documented in table 2.6.
Table 2.6 Correlations between spirometry and white cell counts and between inflammatory markers and time to next exacerbation. Correlations were performed using Spearman’s rank correlation co-efficient.

**Body Mass Index Changes During Treatment**

Body mass index (BMI) was measured at the beginning and end of antibiotic treatment (Table 2.7). BMI at the start of the exacerbation was not significantly different between the treatment outcome groups. Equally there were no significant changes in BMI during treatment for any patient group. No relationship was seen between change in BMI and change in FEV₁ during treatment ($r = -0.02$, $p = 0.93$).
Table 2.7 Median BMI values at the start and end of exacerbation treatment and a comparison of groups defined by treatment outcome. Data is presented as median values and interquartile range.

Quality of Life Scores

Correlations between Quality of Life Scores and Spirometry Values

There were no significant correlations between CFQ-R respiratory domain score and spirometry values (FEV₁, FEV₁% predicted or FEV₁ as percentage of baseline) at day 1 of treatment. There was a mild but significant correlation between FEV₁ as a percentage of baseline and the respiratory domain score of the CFQ-R when all study days were collated (Spearman r = 0.33, p = 0.003). There were no significant correlations between the BCFQ score and FEV₁ as a percentage of baseline or FEV₁ % predicted.

Changes in Quality Of Life Scores During Antibiotic Treatment

When examining all subjects, there was a significant improvement in the CFQR respiratory domain score between the beginning and end of therapy (median 44 vs. 61, p=0.003) as documented in Table 2.8. When examining different treatment groups there were similar improvements in CFQR respiratory domain scores for both groups.
Table 2.8 CFQ-R respiratory domain score during treatment and comparing treatment outcome groups. * p<0.05 compared with day 1 value.

When examining BCFQ scores in all patients there was a statistical significant decrease in the median score at the end of treatment compared to when treatment was commenced as shown in Table 2.9. This was also seen in the treatment success group but not the treatment failure group. However, there were no significant differences of median scores between the treatment groups at either time point.

Table 2.9 BCFQ score during treatment and comparing treatment outcome groups. * p<0.05 compared with day 1 value

There were no significant correlations between changes in QOL scores and changes in spirometry values during treatment of an exacerbation. As the Brompton Cystic Fibrosis Questionnaire is not yet in widespread use, the BCFQ scores were correlated with the CFQR respiratory domain scores for all measured values. There was a moderate correlation between scores (r= -0.49, p<0.0001)
2.4 Discussion

In this study a number of significant findings have been recognised. A number of patients did not return to baseline lung function at the end of treatment of an exacerbation. This group is characterised by a larger drop in lung function from stable baseline values and a longer interval between the stable measure and the start of antibiotic treatment. When antibiotic treatment is commenced, these patients have similar percentage lung function improvements to those who recovered their baseline lung function. A further finding was that in those patients deemed a treatment success, the majority of lung function improvement is seen within the first 7 days of antibiotic treatment with no significant increase in spirometry measures after this time. These will be discussed in the following sections.

Is the Subject Cohort an Accurate Representation of the CF Population?

Clearly a limiting factor of any exacerbation study, especially one that is only analysing those receiving intravenous antibiotics, could be a skew towards patients who have more severe disease and lower baseline lung function. To some extent this is seen in this study. The median FEV\textsubscript{1} % predicted for the adult CF clinic at RBH is 62.3% (CF Registry data 2012) compared with 48% for this cohort. However it is also possible to use the CF registry to identify only those patients who have received intravenous antibiotics and have chronic PA for each clinic centre. At RBH the median FEV\textsubscript{1} % predicted for such a cohort is 50.2%. Those patients who are phe508del homozygous are not over-represented in this cohort (48% in cohort compared with 50% in the CF registry). It would therefore appear that the subjects recruited to this study were an accurate representation of the CF clinic at the centre involved.

Differences in Baseline Characteristics of Treatment Outcome Groups

Treatment outcomes of success and failure were based on the recovery of FEV\textsubscript{1} to within 90% of baseline and a CRP of less than 30 mg/L. Only one patient failed to have a CRP of
less than 30 mg/L and this subject also failed to improve the FEV$_1$ value to within 90%. 20/27 patients recovered FEV$_1$ back to within 90% of baseline. This is a lower ratio than has been seen in other studies. Sanders et al.$^{92}$ analysed US CF Registry Data and determined that 75% of patients returned to within 90% of baseline.

One of the main aims of the clinical tracking study was to determine if there were any predisposing factors at the time of an exacerbation that could predict treatment success. There were no significant differences in the baseline characteristics between the treatment outcome groups except for age and median time from stable measure to the start of treatment of the exacerbation.

At first examination it is difficult to explain the significantly lower age of the treatment failure group and it may well be an effect of the small numbers in the treatment failure group and a cluster of older people in the treatment success group (6/20 were older than 45 years). The level of statistical significance when comparing the differences in time from stable measure suggests it is unlikely to be explained by small numbers in the treatment failure group. In combination with the significantly larger drop in lung function from baseline to day 1 seen in the treatment failure group, it could be considered that those in the treatment failure group have been unwell for longer before commencing antibiotic therapy. This prolonged time without treatment leads to them having worse lung function at the time of commencing antibiotic therapy. These findings agree broadly with those of Sanders et al.$^{92}$ who found that failure to return to baseline was associated with, among other things, a greater drop in FEV$_1$ at the time of commencing treatment and a longer time from stable baseline measure of lung function. Unfortunately the study did not examine the immediate prodrome to the exacerbation. By utilising a symptom diary, for example, a much clearer indication of when the exacerbation started would have been possible and this would have enabled a clear relationship between duration of symptoms and decline in lung function to have been
determined. If such a relationship did exist then this would have significant implications to the clinical management of CF patients with regards to waiting times for admission to commence antibiotic therapy.

**Antibiotic Choice and Duration**

This study could be criticised for not restricting patients to a single combination of antibiotics. However, studies have previously shown no significant differences in clinical outcomes between the use of meropenem and ceftazidime\(^{93,94}\). This study would concur with these previous findings, demonstrating no effect on lung function improvement or time to next exacerbation following treatment, although it is important to note that antibiotic choice was not randomly allocated.

The appropriate duration of antibiotic therapy for CF exacerbations is a much debated subject with no overall consensus. The aim of the study was to treat patients for 14 days as was routine clinical practice at RBH. However some subjects stopped treatment at 10 days. The results showed no significant difference in lung function changes and time to next exacerbation between those who received 10 days and those who received 14 days. Given the small sample size it is difficult to draw any meaningful conclusion from the data regarding antibiotic duration. Clearly as the patients are going home “early” they are more likely to be well with recovered lung function and those with lower baseline lung function were likely to have been given longer courses of antibiotics. To determine the number of patients required in a study to give adequate power to show equivalence of durations of treatment, a power calculation was performed. Assuming that equivalence is a difference in median improvement in FEV\(_1\) of ≤10%, 136 subjects would be required in each group to determine equivalence (significance 5%, power 90%) (If the FEV\(_1\) difference is reduced to 5%, 542 subjects would be required in each group).
Spirometry Measures During Antibiotic Therapy

The question of antibiotic duration could also be debated when lung function responses during treatment are examined. When the entire cohort was analysed it was clear that, as would be expected, there were significant decreases in spirometry values from stable baseline to the start of antibiotic therapy. Perhaps more interesting is the finding that in the treatment success group, lung function was back to baseline values by day 7 and no significant improvement in lung function was seen after this date. Those patients who did not return to baseline did not show a significant improvement in lung function at day 7 but did show some improvement by the end of treatment. The spirometry measures taken at the stable time post treatment were affected by relatively low numbers with patients not attending for the follow-up visit. This is not the first to show a plateau of spirometry improvement. Collaco et al.\textsuperscript{95} have previously shown a similar occurrence with no significant improvements in FEV\textsubscript{1} after 8-10 days of treatment.

A comparison of treatment outcome groups revealed no significant differences between groups for measures of FEV\textsubscript{1} and FEV\textsubscript{1} % predicted for any time points. As treatment outcome was determined by an FEV\textsubscript{1} % OBL of greater than 90%, it is unsurprising that this measure was significantly different between the groups for each treatment time-point. It should also be noted that in the treatment success group, the median FEV\textsubscript{1} %OBL was 89% (and some patient would fit the criteria for treatment success at the start of treatment with FEV\textsubscript{1} %OBL greater than 90% and CRP <30 mg/L).

Perhaps more informative are the analyses of changes in lung function between the treatment groups. The treatment failure group had a significantly greater drop in all spirometry measures between the baseline values and the day 1 values. There were no significant differences between the groups when lung function response to treatment was analysed. It
would therefore seem that those patients who failed to return to baseline lung values did so because of a larger drop in lung function prior to treatment rather than a failure to respond to antibiotic therapy. It is unfortunately beyond the remit of this study to determine whether a change in antibiotics would lead to further lung function improvement. It does however raise an interesting question regarding antibiotic duration. If baseline lung function is recovered within 7 days of treatment for those patients who are going to achieve a return to baseline, would it be appropriate to stop antibiotic therapy at this point? Of course, lung function recovery is not the only factor to be considered and it may well be that time to next exacerbation would be significantly affected by shorter courses of antibiotics. Such questions need to be answered in a larger study.

**Inflammatory markers**

The measurement of inflammatory markers is routine in clinical practice. This study examined CRP, total white cell count and neutrophil count during CF exacerbations. CRP is a sensitive marker for systemic inflammation. Only 13 of the 27 patients had a CRP above the normal limit at the start of therapy and the median level of all subject values was 10 mg/L, which is within normal limits. The median CRP in the treatment failure group was higher than the success group to a level that would be considered clinically significant but it failed to reach statistical significance. This finding would be in keeping with the treatment failure group being generally more unwell at the beginning of the exacerbation, rather than failing to respond to treatment. Total white cell count and neutrophil count were not significantly different between the groups and are perhaps less discriminating for systemic inflammation.

The lack of significant correlation between changes in spirometry and changes in inflammatory markers that was noted is likely related to the levels being generally within the normal ranges.
BMI

Body mass index (BMI) was shown to be relatively unaffected by treatment for an exacerbation with no significant changes seen in either treatment outcome groups. This is perhaps not surprising given the relatively short duration of antibiotic therapy.

Patient Reported Outcome Measures

Patient reported outcome measures such as quality of life questionnaires are being increasingly used in clinical research. In CF the most widely used is the Cystic Fibrosis Questionnaire-revised (CFQR). The questionnaire has a number of symptom domains, but this study examined only the respiratory domain score. The Royal Brompton Hospital CF Department has devised a shorter questionnaire, the Brompton CF Questionnaire (BCFQ) which gives a total score but combines a number of symptom domains. It is important to note that neither questionnaire was designed to be used during an exacerbation, with many questions asking about chronic symptoms. Despite this however, significant changes were seen between the beginning and end of antibiotic therapy for both questionnaires. Scores were not significantly different between treatment outcome groups. This leads to the interesting observation that although the patients have bigger drops in lung function, they did not feel more unwell as determined by symptom score. This however may be an effect of the potential lack of sensitivity of the questionnaires to pick up severity of an exacerbation, something they were neither designed nor validated for in this clinical scenario. An important validation of the BCFQ was the significant correlation between CFQR respiratory domain and BCFQ scores. A higher degree of correlation was likely not seen as the BCFQ includes all symptom domains.
Treatment Outcomes

This study utilised the definition of a treatment success as a return to within 90% of the baseline FEV₁ and a CRP of less than 30. The CRP was less than 30 in all but one patient (who also had an inadequate lung function response). This definition is based on our clinical practice rather than a validated measure of “treatment success”. At present no such measure exists with clinical studies concerning CF exacerbations often suggesting treatment success as decided by the clinician. It is therefore important not to place too much weight on the comparison of treatment outcome groups. In fact as this study shows, there was no significant difference in the improvement in lung function during treatment between the groups. More correctly our treatment failure group is not a “failure to respond to treatment” group but a group who were more unwell at the start of treatment of an exacerbation. In light of this all subsequent analyses were also performed as correlations between changes in the analysed variable with changes in spirometry values.

In conclusion, it has been demonstrated that this cohort of patients is a reasonable representation of the adult CF population. A significant proportion of patients have spirometry values that do not return to baseline at the end of a course of intravenous antibiotic therapy. It has been shown that this is due to a larger decline in lung function prior to commencement of antibiotics rather than a failure to respond to treatment. Subjects who returned lung function back to baseline did so within 7 days of treatment. This may indicate that shorter antibiotic course than are widely used at present may be equally effective.

Serum inflammatory markers and quality of life questionnaires have not been demonstrated to be effective at determining treatment response and do not correlate with spirometry measures.
Having demonstrated the clinical variables during treatment of an exacerbation the following chapters will examine microbiological aspects of the CF exacerbation, and comparing with the data collected in this study. This will involve an examination of the culture based microbiological assays to examine the relationship of PA mucoidy, bacterial density and the presence of other organisms with clinical measures of treatment response. The role of PA hypermutators in exacerbation severity and treatment response will also be examined.

An in-depth examination of the CF microbiome using 16S rRNA gene sequencing to identify bacteria will then explore the effect of antibiotic therapy on the diversity of the bacteria present and if changes in diversity have clinical effects. Finally, an examination of quorum sensing molecules during antibiotic treatment will explore the role of cell-to-cell communication at the time of exacerbation and whether antibiotic therapy has any effect on these systems.
CHAPTER 3

THE CF INFECTIVE EXACERBATION – A MICROBIOLOGICAL STUDY

3.1 Introduction

The majority of microbiology input into CF care is based on routine culture of sputum samples on selective media plates. As well as providing information regarding the bacteria present, routine culture can be used to calculate bacterial density within sputum samples and cultures can be tested against antibiotics to determine \textit{in vitro} bacterial resistance to therapy. This study will examine sputum samples collected during the previous clinical tracking study and examine the microbiological response to exacerbation and treatment by comparing with the previous obtained clinical measures.

Aims of the microbiological study

1. To determine the effect of \textit{Pseudomonas aeruginosa} (PA) mucoidy on clinical outcome measures of lung function and inflammatory markers.
2. To determine whether PA bacterial density is affected by antibiotic treatment.
3. To determine whether resistance to the treatment antibiotic affects treatment outcomes.
4. To determine whether the presence of bacteria cultured in addition to PA has an effect on clinical outcome measures.
5. To determine whether the emergence of \textit{Candida albicans} is associated with worse clinical outcome in antibiotic treatment.
3.2 Methods

Sputum samples were collected as described in the previous chapter. Sputum cultures were processed and reported by the Microbiology Department, Royal Brompton Hospital, following the laboratory protocol for routine clinical processing of CF sputum samples. As described in more detail below, sputum samples underwent homogenisation prior to inoculation of culture plates. After 24-48 hours of incubation, PA isolates underwent antibiotic sensitivity testing.

Sputum homogenisation and dilution

An equal volume of Ringer’s solution containing approximately 10 sterile glass beads (4mm diameter) was added to sputum and vortexed to emulsify giving a 1:2 dilution of sputum. Further dilutions with Ringer’s solution were performed to give a final dilution of 1:1000 sputum homogenate

Inoculation of microbiology plates

Sputum homogenate was inoculated onto non-selective and selective growth media plates for incubation. 20μl of the 1:1000 dilution sputum homogenate was spread over a chocolate agar plate (a non-selective growth medium) and then incubated in CO₂ at 37°C. 20 μl of 1:2 dilution sputum homogenate was spread over 6 other selective media plates and incubated at 37°C in aerobic conditions:

(i) Difco *Pseudomonas* agar plate (enhances pyocyanin production to allow identification of PA),

(ii) MacConkey agar plate (selective media for Gram negative organisms and stains for lactose fermenters such as *E.Coli, Klebsiella sp*),
(iii) *B. cepacia* agar plate (contains antibiotics to allow selective growth of *B. cepacia* and contains an indicator dye which turns the agar pink in the presence of *B. cepacia*),

(iv) Sabouraud’s agar plate (selective fungal growth, low pH inhibits bacterial growth),

(v) Dermasel plate (selective dermatophyte fungal growth),

(vi) Mannitol salt agar plate (selective growth for Gram positive organisms and contains indicator for coagulase reaction).

All plates were examined and colony counting was performed after 48 hours of incubation. Bacterial identification was based on morphological appearances, growth on selective media and use of API-20E (bioMerieux Clinical Diagnostics, France) biochemical reaction kits.\(^9^6\)

PA mucoidy was determined by visual appearance of colonies by experienced microbiology technicians. PA colony forming units per millilitre of sputum (cfu/ml) were calculated from cultured sputum samples for each point study visit. The total cfu/ml was calculated combining all morphotypes of PA.

**Antibiotic sensitivity testing – BSAC Disc Diffusion Method**

Antimicrobial susceptibility testing of PA was performed using the disc diffusion method as recommended by the British Society for Antimicrobial Chemotherapy (BSAC)\(^9^7\).

Using a sterile loop, at least 4 four morphologically similar colonies were transferred to 4ml of sensitivity broth (ISO-sensitest broth, Oxoid Ltd, Cambridge). The broth was then placed in a shaking incubator at 37°C until the visible turbidity was equal to or greater than 0.5 McFarland standard. The inoculum was spread evenly over the surface of an ISO-sensitest agar plate (Oxoid, Cambridge). Within 15 minutes of inoculation antimicrobial discs were applied to the surface of the plate with a maximum of 6 discs per plate and then incubated for 18-20 hours at 37°C. PA organisms were tested against the following antibiotics: amikacin,
azithromycin, aztreonam, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, temocillin, timentin and tobramycin.

After incubation plates were read using the OXOID AURA automated zone reader (Oxoid, Cambridge) to measure zone diameters and interpret sensitivity results.

**Liverpool Epidemic Strain typing**

To ensure results were not skewed by the presence of an epidemic strain of PA, which have been shown to have increased virulence and transmissibility\(^{98,99}\), PA isolates were also assayed for the presence of Liverpool Epidemic Strain (LES) PA. This was kindly performed by the HPA Laboratory, Cambridge using an in-house multiplex PCR assay to detect the suppression subtractive hybridisation (SSH) sequences PS21 and F9 in bacterial DNA which have previously been identified as being specific to LES PA\(^{100}\).

**Statistical methodology**

Statistical analysis was performed as described in the methods section of Chapter 2.
3.3 Results

Of the 27 patients included in the analyses, 12 cultured only a mucoid phenotype of PA, 8 cultured only non-mucoid PA, with the remaining 7 culturing both mucoid and non-mucoid morphotypes of PA. For the purposes of analysis those isolating both mucoid and non-mucoid morphotypes have been grouped with those isolating the mucoid phenotype.

Does PA morphotype affect clinical measures?

There were no significant differences between morphotype groups for day 1 median values of FEV₁, FEV₁ % predicted or FEV₁ as a % of baseline, CRP, or total white cell count (Table 3.1). There was no significant difference in the median age of subjects isolating mucoid morphotypes as compared with non-mucoid (mucoid 33 yrs (25,43); non-mucoid 29 yrs (18-46); p = 0.51)

Using a Fishers exact test, the distribution of morphotypes between treatment outcomes (success or failure, as described in the previous chapter) was examined. There were no significant differences between the relative number of subjects isolating the mucoid morphotype versus the non-mucoid morphotype in either treatment outcome group at any of the study time points (p values; day 1, 1.0; day 7, 0.38; end of treatment, 0.11).

There were no differences between the groups when comparing the morphotypes present at day 1 and the overall improvement in FEV₁ (Table 3.1 and Figures 3.1 and 3.2)
Table 3.1  Day 1 spirometry values and inflammatory markers comparing non-mucoid and mucoid morphotypes of PA

Changes in spirometry during treatment comparing non-mucoid and mucoid morphotypes of PA

![Image of box and whisker plots for CRP and WCC](image)

Figure 3.1  Day 1 inflammatory markers comparing non-mucoid and mucoid morphotypes of PA. Data is presented as box and whisker plots with boxes representing median and interquartile ranges and the whiskers representing maximum and minimum values. No significant differences were seen for CRP or white cell count.
Figure 3.2 (a) Day 1 spirometry values comparing non-mucoid and mucoid morphotypes of PA (b) change in spirometry (FEV₁ improvement and %FEV₁ improvement) comparing morphotypes. Data is presented as box and whisker plots with boxes representing median and interquartile ranges and the whiskers representing maximum and minimum values. No significant differences were seen for any of the spirometry measures.
Is PA Bacterial Density Affected by Antibiotic Treatment?

The median PA bacterial density on day 1, prior to commencement of antibiotics, was $7.3 \times 10^7$ cfu/ml ($6.8 \times 10^6$, $7.3 \times 10^8$). There were no significant relationships between spirometry values on day 1 of treatment with bacterial density as detailed in table 3.2.

<table>
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<th>Correlations</th>
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<th>P value</th>
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<tr>
<td><strong>Day 1 Spirometry vs. PA cfu/ml</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV$_1$</td>
<td>-0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>FEV$_1$ % predicted</td>
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<td>0.83</td>
</tr>
<tr>
<td>FEV$_1$ % OBL</td>
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</tr>
</tbody>
</table>

Table 3.2  Correlations between day 1 spirometry values and PA bacterial numbers (cfu/ml). No significant correlations are seen. Correlations are performed using Spearman’s rank correlation co-efficient.

There was a greater density of bacteria in the treatment failure group but this did not reach statistical significance ($4.5 \times 10^7$ vs. $4.5 \times 10^8$, p=0.24) as represented in figure 3.3.

In the total study population there were no significant differences between PA cfu/ml prior to starting therapy compared with the end of therapy or the stable post therapy measure as demonstrated in Table 3.3 and Figure 3.4. No difference was seen in the change in PA cfu/ml between the treatment success and failure groups (success: $-3.3 \times 10^8$, failure: $-2.9 \times 10^6$, p=0.40).
Figure 3.3  PA CFU/ml prior to commencing therapy comparing treatment outcomes. Data is presented as box and whisker plots with the boxes representing median values and interquartile ranges. The whiskers represent maximum and minimum values. No significant difference in PA bacterial density is seen between treatment outcome groups.
Table 3.3 The change in PA bacterial numbers during treatment and comparing treatment outcome groups. No comparison was possible for the stable post treatment values due to insufficient numbers of subjects in the treatment failure group.

<table>
<thead>
<tr>
<th></th>
<th>All (n=27)</th>
<th>Rx Success (n=20)</th>
<th>Rx Failure (n=7)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PA cfu/ml median</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>7.3 x 10^7</td>
<td>4.5 x 10^7</td>
<td>4.5 x 10^8</td>
<td>0.24</td>
</tr>
<tr>
<td>(5.2x10^6,7.1x10^7)</td>
<td>(1.7x10^7,1.8x10^8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>2.8 x 10^7</td>
<td>4.0 x 10^7</td>
<td>2.7 x 10^7</td>
<td>0.12</td>
</tr>
<tr>
<td>(2.5x10^6,3.3x10^7)</td>
<td>(0, 2.1x10^8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>End Rx</td>
<td>1.5 x 10^7</td>
<td>1.5 x 10^7</td>
<td>4.7 x 10^8</td>
<td>0.96</td>
</tr>
<tr>
<td>(1.6x10^6,9.4x10^7)</td>
<td>(2.2x10^6,2.3x10^7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable post</td>
<td>2.4 x 10^6</td>
<td></td>
<td>4.7 x 10^8</td>
<td>0.96</td>
</tr>
<tr>
<td>(1.4x10^5,9.6x10^6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.4 PA bacterial numbers during treatment and comparing treatment outcome groups. Data is presented a histogram representing median values with interquartile ranges represented by error bars. No significant differences are seen between groups for any time point.
The change in PA density was correlated with spirometry changes to determine whether a relationship existed. While there appeared to be a moderate correlation for both absolute FEV$_1$ and percentage change in FEV$_1$, this did not meet statistical significance as shown in table 3.4 and figures 3.5 and 3.6.

<table>
<thead>
<tr>
<th>Correlations</th>
<th>R value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in Spirometry vs. Change in PA cfu/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in absolute FEV$_1$</td>
<td>-0.40</td>
<td>0.08</td>
</tr>
<tr>
<td>%change in FEV$_1$</td>
<td>-0.40</td>
<td>0.09</td>
</tr>
<tr>
<td>End cfu/ml vs. TTNE</td>
<td>-0.01</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Table 3.4 Correlations comparing changes in spirometry measures during antibiotic treatment with changes in PA bacterial numbers.

![Graph](image)

Figure 3.5 The correlation between change in FEV$_1$(L) and the change in PA cfu/ml during antibiotic treatment. Correlation was performed using Spearman’s rank correlation co-efficient. R= -0.40, p=0.08
Figure 3.6 The correlation between change in FEV$_1$ as a percentage and change in PA cfu/ml during antibiotic treatment. Correlation was performed using Spearman’s rank correlation co-efficient, \( R = -0.40, \) \( p = 0.09. \)

Five subjects who cultured PA from their sputum samples at the beginning of treatment failed to isolate PA in sputum culture by the end of treatment. Of these, one subject had both mucoid and non-mucoid phenotypes of PA on day 1 of treatment, 2 had only the mucoid phenotype and 2 had only the non-mucoid phenotype. There was no relationship between clearance of PA and baseline lung function, change in lung function during treatment of the exacerbation, end of treatment CRP or antibiotic choice. This may not represent eradication of the bacteria as the limit of detection for culture is \( 10^3 \) cfu/ml

**Antibiotic Sensitivity and Treatment Response**

Eight patients isolated PA on day 1 that exhibited *in-vitro* resistant to one of the antibiotics being used to treat the exacerbation. One patient isolated PA that exhibited *in-vitro* resistance to both antibiotics used. During antibiotic treatment, no PA isolates developed resistance to those antibiotics being used.
The presence of antibiotic resistance from day 1 had no effect on FEV$_1$ improvement during treatment (% improvement in FEV$_1$, fully sensitive 21 (14, 37), resistant 13 (6, 45) p= 0.52) or CRP at the end of treatment (sensitive 3.5 (1, 5.8) vs. resistant 6 (1,15) p= 0.3) as documented in Table 3.5 and Figure 3.7

When examining bacterial numbers, those subjects with an antibiotic resistant organism had significantly higher bacterial levels at the beginning and end of treatment but the change in bacterial numbers during treatment were not significantly different between groups.

All subjects that failed to culture PA on routine microbiological testing during treatment had initially isolates that were fully sensitive to the antibiotic therapy.

<table>
<thead>
<tr>
<th></th>
<th>Fully sensitive bacteria</th>
<th>Presence of antibiotic resistance</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV$_1$ % change during treatment</td>
<td>21 (14,37)</td>
<td>13(6, 45)</td>
<td>0.52</td>
</tr>
<tr>
<td>CRP at end of treatment (mg/L)</td>
<td>3.5 (1,5.8)</td>
<td>6 (1,15)</td>
<td>0.3</td>
</tr>
<tr>
<td>PA cfu/ml start of treatment</td>
<td>$2.1 \times 10^7$</td>
<td>$7.4 \times 10^8$</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>(2.1 x $10^6$, 3.3 x $10^8$)</td>
<td>(8.9 x $10^7$, 1.5 x $10^8$)</td>
<td></td>
</tr>
<tr>
<td>PA cfu/ml end of treatment</td>
<td>730,000</td>
<td>$2.1 \times 10^7$</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>(0, 2.6 x $10^7$)</td>
<td>(5.0 x $10^6$, 1.1 x $10^6$)</td>
<td></td>
</tr>
<tr>
<td>Change in PA cfu/ml</td>
<td>$-1.2 \times 10^7$</td>
<td>$-2.2 \times 10^8$</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>(-2.7 x $10^6$,8.9 x $10^6$)</td>
<td>(-6.1 x $10^7$, 1.9 x $10^8$)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5 A comparison of spirometry and bacterial density changes during antibiotic treatment between groups defined by the presence or absence of PA bacteria resistant to at least one of the antibiotic used in treatment.
**Liverpool Epidemic Strain *Pseudomonas Aeruginosa***

PCR assays of DNA extracted from PA isolates were performed to determine the presence of Liverpool Epidemic Strain (LES) PA. Two subjects were found to isolate the LES PA. Both of these patients were in the treatment failure group and both isolated PA that was resistant *in-vitro* to one of the antibiotics used to treat the exacerbation.

**Co-existent Bacteria**

In nine subjects *Staphylococcus aureus* (SA) was cultured from sputum samples at the beginning of the exacerbation, these were all methicillin-sensitive (MSSA) strains. Two subjects cultured a fungal species, one patient grew *Aspergillus fumigatus* and the other *Scedosporium sp*. No subjects cultured *Achromobacter xylosidans*, *Stenotrophomonas maltophilia* or *Burkholderia* species.

There was no significant difference between those subjects who cultured SA at the beginning of an exacerbation when comparing FEV₁ (1.38 litres in those with SA, 1.19 in those without,
p = 0.13), FEV$_1$ %pred (42.0 vs. 38.0, p=0.71), FEV$_1$ as a % of baseline (74.3 vs. 88.2, p=0.36) or CRP (8.5 vs. 16.0, p=0.29) (Figure 3.8). The presence of SA at the beginning of treatment had no effect on treatment success or overall FEV$_1$ improvement. There was a trend to subjects who isolated SA to have a lower median age, but this did not reach significance (SA present, median age 26 (21, 24), SA absent, median age 37 (28, 47), p=0.08)

![Graphs](image)

Figure 3.8 Graphs representing median values of spirometry measures in groups defined by the presence or absence of SA on bacterial culture. Data is presented as box and whisker plots with the boxes representing median values and interquartile ranges. The whiskers represent maximum and minimum values. No significant differences are seen between groups for any measure.
Isolation of Candida Albicans

Fifteen subjects (55%) isolated Candida Albicans from sputum culture while receiving antibiotic therapy. The isolation of Candida was not associated with differences in FEV$_1$ improvement or CRP. Candida isolation was associated with a greater treatment-related decline in PA bacterial density but this did not reach statistical significance (Table 3.6). Candida was not isolated more commonly in those patients who failed to isolate PA during treatment. There were no relationships between the isolation of Candida and the use of steroids, the use of meropenem, or the presence of hypermutators. There were no significant differences between treatment outcome groups with regard to Candida isolation.

<table>
<thead>
<tr>
<th></th>
<th>Candida Albicans Isolated (n=15)</th>
<th>No Candida Isolated (n=12)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV$_1$ % change during treatment</td>
<td>16 (7,37)</td>
<td>20 (9,37)</td>
<td>0.68</td>
</tr>
<tr>
<td>Change in PA cfu/ml</td>
<td>-2.4 x 10$^7$ (-6.6 x 10$^8$, -3 x 10$^6$)</td>
<td>-1.0 x 10$^7$ (-1.1 x 10$^8$, 1.4 x 10$^6$)</td>
<td>0.07</td>
</tr>
<tr>
<td>CRP at end of Rx</td>
<td>4 (1,7)</td>
<td>2.5 (1,7)</td>
<td>0.77</td>
</tr>
<tr>
<td>PA Hypermutators present</td>
<td>8</td>
<td>4</td>
<td>0.68</td>
</tr>
<tr>
<td>Meropenem use</td>
<td>5</td>
<td>8</td>
<td>0.13</td>
</tr>
<tr>
<td>Treatment success</td>
<td>12</td>
<td>8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 3.6 The clinical effect and associations with isolation of Candida albicans in sputum culture. Continuous variables p value determined by Mann-Whitney test. Categorical variable p values determined by Fisher exact test.
3.4 Discussion

This analysis of the microbiological changes seen at the time of exacerbation and subsequent treatment has shown that severity of exacerbation is not related to the PA bacterial numbers and that, in this small cohort, bacterial numbers do not change significantly during treatment. PA mucoidy was not shown to have a significant effect on exacerbation severity or treatment response. The presence of bacterial resistance to the antibiotic treatment used did not appear to affect treatment responses but the resistant bacteria were present in greater numbers. The only other bacterium present in significant numbers was *Staphylococcus aureus* but its presence did not appear to relate to exacerbation severity or treatment response. In a significant number of patients *Candida albicans* emerged in sputum cultures during treatment. This was not associated with any clinical outcome measure but there was a trend to lower PA bacterial numbers in those patients who isolated *Candida*.

Mucoidy

In this study the presence of mucoid morphotypes of PA had no significant effect on any clinical markers of severity of the exacerbations. Is this a surprising finding? Alginate production in PA is complex and involves the interplay of a number of genes. The conversion to a mucoid phenotype has been shown to be related to genetic mutation, most commonly in the *mucA* gene and is thought to be a response to the host environment, for example oxygen radicals produced by neutrophils as an immune response. A longitudinal study of CF children has shown a median time of transition from non-mucoid to mucoid PA infection of 10.9 years. The study also demonstrated acquisition of the mucoid phenotype was associated with a greater decline in lung function. However, that study examined chronic changes in spirometry. No study to date has addressed the difference in outcomes of exacerbations comparing mucoid and non-mucoid PA.
In this study the first sample was collected at a time of exacerbation rather than during a stable clinical state and it could be that the balance of non-mucoid versus mucoid PA is very different at the time of an exacerbation. If Sauer’s biofilm stages\textsuperscript{103} are to be considered, exacerbation may be occurring at Stage 5, the dispersal stage where there are more likely to be motile, non-mucoid PA released from the biofilm. In the study, those subjects isolating both mucoid and non-mucoid strains were included with the mucoid-only group for analysis. It could be argued that they represent a separate group and should have been analysed accordingly, but small numbers would have made the interpretation of findings difficult.

**Quantitative Microbiology**

Whether there is a change in PA bacterial density during antibiotic treatment is a contentious issue. A previous study has suggested that bacterial numbers do not increase in the immediate prodrome of infection\textsuperscript{104}. During antibiotic treatment, studies have shown conflicting data with some showing a significant decrease in bacterial numbers\textsuperscript{29,52,105} and others no significant change in bacterial numbers\textsuperscript{30,106}. In this study overall bacterial numbers did not change significantly between the beginning and end of therapy. Those in the treatment failure group (who have been identified as having a greater decline in lung function from the pre-exacerbation stable value) had a log\textsubscript{10} greater median PA bacterial density at the start of antibiotic therapy, but this was not a statistically significant finding. Equally no significant changes were seen between the treatment outcome groups at any of the time points. As previously discussed however, the patients in both groups improved clinically and so it may be more informative to look at absolute changes in lung function and their relationship with bacterial density.

There was no correlation between day 1 spirometry measures and PA bacterial density. However when the relationship between change in spirometry and change in PA bacterial
density was correlated, there was a moderate negative correlation seen which did not quite reach statistical significance. It would appear therefore that there may be some relationship between bacterial killing and improvement in lung function. This is in disagreement with a previous study which showed no relationship between lung function and bacterial density changes during IV antibiotics. To determine whether these current study findings are true would require an adequately powered study, examining this question specifically.

**Clearance of PA bacteria**

All the subjects included in the study had chronic PA infection which had been defined as at least 2 positive cultures in the preceding 6 months. It was therefore a surprising finding to see 20% of patients failing to isolate PA in their sputum cultures by the end of treatment. It is unlikely this is true eradication as the limit of detection for our PA culture was $1 \times 10^3$ cfu/ml. It may also be an effect of antibiotics in the sputum samples inhibiting growth on the selective media plate, or sputum volumes decreasing. It was not limited to only those patients isolating non-mucoid strains of PA and it had no relationship with clinical outcomes. A further examination of this finding will be discussed with the results of the 16S rRNA gene sequencing method of bacterial identification in Chapter 5.

**Antibiotic Resistance and Treatment Response**

The presence of antibiotic resistance had no effect on treatment response in terms of spirometry improvement and changes in inflammatory markers. However, the PA bacterial density was higher in the patients with resistant organisms at both the beginning and end of treatment. No significant differences were seen in the change in bacterial density during treatment when comparing fully sensitive and resistant isolates, although this echoes the previous result of no significant changes in bacteria during an exacerbation. Previous studies examining PA antibiotic resistance have examined multiple-antibiotic-resistant PA(MARPA),
which is defined as PA resistant to all antibiotics in at least 2 of the following classes: quinolones, beta-lactams and aminoglycosides\textsuperscript{108}. Studies of subjects with these strains of PA have shown an association with more severe lung disease and a more rapid rate of lung function decline\textsuperscript{109;110}. In the current study, data was only collected on resistance to the antibiotic used to treat the exacerbation rather than multiple resistances. The data is therefore not comparable. Equally the study reported by Foweraker et al.\textsuperscript{111} should be borne in mind when interpreting this data. These authors have shown that antibiotic sensitivity testing is highly variable and not necessarily reproducible. Those organisms with some degree of antibiotic resistance appear to persist at higher numbers in the CF airway regardless of treatment.

**Liverpool Epidemic Strain**

Epidemic strains of PA such as the Liverpool Epidemic Strain (LES) have been shown to be more virulent with increased transmissibility\textsuperscript{98;99}. It would therefore have affected the validity of the clinical data if large numbers of the cohort isolated such strains. The study revealed only 2 subjects with LES isolates; both isolated PA with resistance to the treatment antibiotic used and both subjects were in the treatment failure group. The LES isolate subjects are too low in number to make any meaningful comment on the effect of treatment of the exacerbation. A potential weakness of the study would be not testing for other epidemic strains. However, while other epidemic strains exist in the CF community, strain typing in the RBH patient cohort has not identified it as a significant issue.

**Co-existent Bacteria and other Organisms**

The routine clinical culture of sputum samples in CF care involves the use of selective media to isolate only what are thought to be potentially pathogenic bacteria. In this study therefore the isolation of other bacteria was very selective and perhaps does not give a true view of all
the bacteria that could be isolated from the sputum samples. However the microbiome will be examined in more detail for its diversity in a subsequent chapter. Clearly *Staphylococcus aureus* (SA) is a commonly isolated organism in CF, especially in younger individuals. In this study, co-isolation of SA study did not appear to have any effect on clinical measures. Previous studies have shown an effect of co-infection with SA and PA. Rosenbluth et al. identified co-infection as a significant risk factor for rapidly declining lung function\(^{112}\). A much smaller study in children also showed an increased mortality rate in subjects with PA and SA infection compared with SA or PA infection alone\(^{113}\). The numbers of co-infected subjects were very small (7/81) and the 10 yr survival estimate of 57% in the co-infected group compared with 92-100% in other groups seems unfeasible low. The results of this study should therefore be interpreted with caution.

The chronic isolation of *Candida albicans* has previously been shown to be associated with a greater rate of decline in FEV\(_1\) and more frequent hospital-treated exacerbations\(^{114}\), although whether this is a causal relationship is not clear. Its presence in patients with severe deficits in FEV\(_1\) may represent the extra antibiotic burden in these patients. In the current study, 55% of patients isolated *Candida* during antibiotic treatment. This is perhaps not the same clinical situation as those patients chronically isolating *Candida*, and also possibly represents oropharyngeal candidiasis contaminating sputum samples. However there did appear to be an association with *Candida* isolation and a greater decline in PA bacterial density (although this did not reach statistical significance). No other clinical marker appeared to be affected by *Candida* isolation, but it may be that it represents a surrogate marker for a decline in pathogenic organisms, allowing other organisms to predominate.

In summary, the presence of PA mucoid morphotypes does not appear to have a significant effect on exacerbation severity or treatment response. PA bacterial density does not change significantly during treatment although a trend towards a relationship between lung function
change and the change in PA bacterial density was seen. The co-isolation of SA in sputum culture had no effect on treatment outcomes or exacerbation severity. The emergence of *Candida albicans* during treatment was associated with a greater decline in PA bacterial density but did not have an effect on treatment outcomes.

If PA bacterial numbers do not change significantly but patients clinically improve, is a change occurring in the expression of virulence factors of PA that is being switched on at the time of exacerbation and switched off during treatment? Or does the overall diversity of the bacteria present have an effect on treatment outcomes. PA hypermutators allow the rapid acquisition of virulence in PA populations. The following chapters will examine the role of hypermutator strains of PA and their role in infective exacerbations, the role of bacterial diversity as determined by bacterial identification through gene sequencing and finally an examination of QS molecule production in PA, to determine what, if any, this system has on exacerbation severity and treatment response.
CHAPTER 4

HYPERMUTATOR STRAINS OF PSEUDOMONAS AERUGINOSA – THEIR ROLE IN CF EXACERBATIONS

4.1 Introduction

Hypermutator phenotypes of Pseudomonas aeruginosa have been noted to be present in significant numbers in CF sputum samples. The relative abundance of hypermutator phenotypes has been shown to increase with duration of chronic infection\textsuperscript{115} and its presence has been shown to be associated with poorer lung function\textsuperscript{116}. Their presence has been shown to be associated with increased antibiotic resistance \textit{in vitro}\textsuperscript{115} and in a mouse model\textsuperscript{117}. No clinical study has shown the effect of its presence on the severity of an exacerbation or the response to antibiotic treatment.

Aims of the Hypermutator study

1. To determine if the presence of hypermutator strains of PA is associated with a more severe clinical state.

2. To determine if the presence of hypermutator strains of PA is associated with a poorer response to antibiotic therapy.
4.2 Methods

Sputum samples for this hypermutator assay were collected at the beginning and end of the IV antibiotic treatment course.

Sputum was homogenised as per the microbiology assay described in the previous chapter and plated onto chocolate agar and *Pseudomonas* plates and incubated at 37°C in CO2 for 24-48 hours.

*P. aeruginosa* was identified by morphological appearances and/or by API reaction kit.

Isolates of each morphotype of PA were sub-cultured along with 2 control organisms (PA01 and PA01 *mut S*, a control hypermutator strain of PA) onto half blood agar and re-incubated at 37°C in CO2. After 24 hours 3-5 bacterial colonies of each isolate were suspended in 15ml of Muller Hinton Broth and incubated overnight at 37°C in a rocking incubator at 140rpm.

After incubation, broth samples were examined for opacity, a heavy swirl suggestive of >10^8 cfu/ml. Samples with lesser opacity were centrifuged at 4000 rpm for 10 minutes and 5 ml of supernatant removed. The remaining 10ml was vortexed and the supernatant added back until the desired opacity was reached.

2ml of each isolate broth was removed and pooled.

Serial tenfold dilutions with PBS were then made of the pooled and controlled samples to give final concentrations of >10^3 cfu/ml of pooled and *mutS* control samples, and >10^2 cfu/ml of PA01 control samples.

Rifampicin-incorporated plates and MHA plates were then inoculated in triplicate using a spiral plater. 50ul and 200ul of each of the diluted pooled broth and control sample broths were plated onto rifampicin-incorporated plates and 50 μl of each sample were inoculated onto the MHA plates.
Plates were then incubated at 37°C, with colony counts performed at 24 and 48 hours. Colony counts were calculated after allowing for volume and dilution of samples.

Mutation frequency was calculated as the mean growth on rifampicin incorporated plates divided by the mean growth on MHA plates.

An isolate was considered to contain a hypermutator strain if the mutation frequency was more than 20 times greater than that of the PA01 control organism\textsuperscript{118}. For analysis, only the presence or absence of a hypermutator strain in each sputum sample was considered.

Statistical analysis was performed as described in Chapter 2 methods.
4.3 Results

Data from 24 patients were available for analysis. Twelve subjects (50%) of patients had PA hypermutator strains isolated from sputum prior to commencing antibiotic therapy.

There was a trend to those subjects isolating hypermutator strains being older than those who didn’t isolate them but this did not reach statistical significance (hypermutator strain present, 41years (30,46); hypermutator strain absent 27 years (23,20), p=0.05) as documented in Table 4.1

The presence of hypermutator strains was not associated with a significant difference in FEV1 % predicted at the start of the exacerbations. The median FEV1 %OBL was higher in those with hypermutator strains present but these failed to reach statistical significance. The CRP median values were not significantly different between the groups. Using a Fishers exact test there was a trend to more hypermutator strains in the treatment success group (11/17) versus the treatment failure group (1/6) (p=0.07).

<table>
<thead>
<tr>
<th>Day 1 Values</th>
<th>Hypermutator strain absent</th>
<th>Hypermutator strain present</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>27 (23,30)</td>
<td>41 (30,46)</td>
<td>0.05</td>
</tr>
<tr>
<td>FEV1 % pred</td>
<td>38 (26,47)</td>
<td>41 (25,48)</td>
<td>0.69</td>
</tr>
<tr>
<td>FEV1 as % of baseline</td>
<td>73 (59,89)</td>
<td>88 (77,94)</td>
<td>0.07</td>
</tr>
<tr>
<td>CRP</td>
<td>23 (9,38)</td>
<td>9 (3,24)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Table 4.1 A comparison of clinical measures on day 1 of antibiotic treatment in groups defined by the presence or absence of hypermutator strains of PA.
16 patients had matched data for hypermutator samples at the beginning and end of treatment. Of these, three subjects isolated hypermutator strains only at the end of treatment. No patient who isolated hypermutator strains at the start of treatment failed to do so at the end of treatment.

In those patients who isolated hypermutator strains at day 1, the median percentage change in FEV$_1$ during treatment was 13% (-1, 31), compared with 27% (15, 49), p=0.07, in those without hypermutator strains as shown in Figure 4.1.

![Figure 4.1](image-url)  
**Figure 4.1** Individual changes in FEV$_1$ %predicted between the start and end of treatment. Those patients with a hypermutator strain (red) had a trend towards a smaller improvement in FEV$_1$ % predicted as than those without a hypermutator strain (blue), 13% vs. 27% (p=0.07).
Effect of Hypermutator Status on Changes in Bacterial Density

There was no association between the presence of hypermutator strains and PA bacterial density at the beginning or end of therapy with no significant differences between the median values. However, the change in PA density between groups was significantly different with a median value of $-2.4 \times 10^8$ in those without hypermutator strains compared with $1.1 \times 10^8$ for those with hypermutator strains ($p=0.02$), Table 4.2 and Figure 4.2.

There was no relationship between antibiotic resistance at day 1 of treatment and the presence of hypermutators, Fisher exact test $p=1.0$.

<table>
<thead>
<tr>
<th></th>
<th>Hypermutator strain absent</th>
<th>Hypermutator strain present</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 PA cfu/ml</td>
<td>$3.3 \times 10^8$</td>
<td>$3.6 \times 10^7$</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>(2.2x10^7, 1.2x10^9)</td>
<td>(6.0x10^6, 6.3x10^8)</td>
<td></td>
</tr>
<tr>
<td>End of Rx PA cfu/ml</td>
<td>$8.2 \times 10^8$</td>
<td>$1.5 \times 10^8$</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>(4.2x10^6, 9.4x10^8)</td>
<td>(9.9x10^6, 7.7x10^8)</td>
<td></td>
</tr>
<tr>
<td>Change in PA cfu/ml</td>
<td>$-2.4 \times 10^8$</td>
<td>$1.1 \times 10^8$</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(-6.1x10^8, -1.2x10^7)</td>
<td>(-3.9x10^7, 8.7x10^8)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2 A comparison of PA bacterial density at the beginning and end of antibiotic therapy and the median change in bacterial density in groups defined by the presence or absence of hypermutator strains of PA.
Figure 4.2 A box and whisker plot comparing the change in PA bacterial density during antibiotic therapy between groups defined by the presence or absence of hypermutator strains of PA. The boxes represent median values and interquartile ranges and the whiskers represent maximum and minimum values. The change in PA bacterial density is significantly higher in the hypermutator negative group $p=0.02$. 
4.4 Discussion

The hypothesis of this study was that hypermutator strains of PA were not significantly more pathogenic but appeared when the PA population was under stress, in this case antibiotic therapy, as a mechanism to survive through a process of rapid beneficial mutation. Has this hypothesis been proved?

Fifty percent of subjects had hypermutator strains present at the commencement of therapy, a frequency consistent with previous studies\textsuperscript{116,119}. The median age of subjects isolating hypermutator strains was not significantly older. This would be in keeping with previous findings of hypermutation being associated with duration of chronic infection with PA, although data in this study was not collected regarding time of first PA infection\textsuperscript{115} and age of subject may not be an appropriate substitute measure. The presence of hypermutators was associated with a trend to a smaller decline in FEV\textsubscript{1} from baseline which did not reach statistical significance (Table 4.1). There was also a trend to more hypermutator strains in the treatment success group, although this is likely representative of the lesser decline in FEV\textsubscript{1} as a percentage of baseline at day 1. The median percentage change in FEV\textsubscript{1} during treatment was higher in those without hypermutator strains but again this is likely to represent a bigger drop in lung function prior to commencing therapy rather than an effect of the hypermutator strains on treatment response. From these results it would appear that hypermutator strains are associated with less severe exacerbations. Previous studies have shown that hypermutator strains are less virulent\textsuperscript{120,121} \textit{in vitro}. The current study data may be consistent with the findings of these studies.

Of the 16 patients who had matched data for the beginning and end of therapy, only 3 acquired new hypermutator strains and no patients lost hypermutator strains during treatment.
This number is too small to determine the effect, if any, of new hypermutator isolation during treatment.

PA bacterial density at the beginning and end of therapy was not significantly affected by the presence of hypermutator strains but an interesting observation was a significantly greater decline in PA density during treatment in the absence of hypermutator strains. This may suggest that hypermutator strains of PA are better able to withstand bacterial killing by antibiotics. Antibiotic resistance did not appear to be significantly more common in subjects with hypermutator strains and no increased antibiotic resistance was seen during treatment in any subjects. Waine et al have previously demonstrated this similar lack of new antibiotic resistance in the presence of hypermutator strains\(^{116}\). The method of analysis does limit the analysis of resistance however. This study is only examining the presence or absence of hypermutator strains. The hypermutator strains themselves were not tested for antibiotic sensitivities directly and it is possible that the resistance profiles are for non-hypermutator strains of PA cultured from the same sample.

This study does have other limitations. Firstly the numbers are small and even smaller for matched data from the beginning and end of treatment. However the percentage of isolates is similar to previous studies so is likely to be representative of the CF population. Age may be a major confounding factor. The clinical tracking study identified an older population in the treatment success group and in this study there was a trend towards an older median age in those who isolated hypermutator strains. It is possible that hypermutator strains are seen more commonly in older patients because, by extrapolation, they have experienced a longer period of chronic PA infection and our findings merely represent older patients with less severe exacerbations. A larger scale study without such a skew in age may answer this more clearly.
As mentioned previously the method of the assay and analysis depending only on presence or absence of hypermutator strains limits the study. It is possible that a subject with 80% of PA in their sputum being hypermutator strains would have different clinical responses to treatment than someone with only 8% of PA being hypermutators.

In summary, hypermutator strains are more prevalent in older patients but may be associated with less severe exacerbations. This would be in keeping with previous work suggesting they are less virulent organisms and act to sustain PA populations by allowing mutations that ensure survival of the bacteria in the CF host, but perhaps at the cost of virulence\textsuperscript{89}. 
5.1 Introduction

The introduction of high throughput gene sequencing has allowed the detection of large numbers of genetic sequences in clinical samples. This technology has been used in CF research to examine the bacterial ecology of the chronic lung infection that is common to all CF patients. Previous work has examined patients who are both stable\textsuperscript{122,123} and exacerbating\textsuperscript{29,30}. The previous exacerbation data has been limited to changes in bacterial diversity alone and has not examined the associated clinical responses. Using 16S rRNA gene sequencing this study will examine in a much greater detail the bacterial diversity of the CF lung during treatment of an exacerbation and by comparing these changes with clinical data will examine whether relationships exist between clinical measures and bacterial diversity.

Aims of the microbiome analysis

To determine if:

1. The bacterial diversity of samples changes during antibiotic therapy.
2. Treatment outcome is related to differences in the effect of treatment on the microbiome.
3. Changes in PA bacterial density is associated with changes in overall bacterial diversity.
4. If antibiotic choice has an effect on bacterial diversity.
5. If bacterial diversity at the end of antibiotic treatment is associated with time to next exacerbation.
5.2 Methods

Extraction of DNA from Sputum Samples

16S rRNA DNA was extracted from sputum samples using the FastDNA SPIN kit for soil (MP Biomedicalls, Ohio, USA), following the manufacturer’s instructions, substituting soil for 750 μl of sputum and using a Precellys 24 homogeniser at 6800rpm for 2 x 30 secs to homogenise the samples. DNA was eluted in 100 μl of ultrapure water.

Measuring Extracted DNA Quantity and Purity

The extracted DNA was tested for quality and quantity using a microvolume spectrophotometer (Nanodrop ND-1000 Spectrophotometer). 2μl of eluted DNA was placed in the spectrophotometer and samples were deemed suitable if the DNA content was greater than 30ng/μl and the purity of the DNA (as measured by ratio of absorbance at wavelengths of 260nm and 280nm) was greater than 1.8.

Eluted DNA was then stored in a -20°C freezer for later analysis.

16S rRNA Gene PCR

DNA PCR, gene sequencing and preparation of the raw sequencing data were performed by members of the Molecular Genetics Group, National Heart and Lung Institute, Imperial College. PCR methods were based on a previously published protocol\textsuperscript{124}.

The V3-V5 hypervariable regions of the 16S rRNA gene were amplified using primer 357F with adaptor B from 454 Life Sciences:

\[ 5' \text{ CTATCCCTGTGTGCTTGGCAGTCTCAGCCTACGGGAGGCAGCAG } 3' \]

and primer 926R which included the 454 Life Sciences adaptor A and a 12 base-pair error-correcting Golay barcode\textsuperscript{125} (denoted with ‘N’), which allows samples to be multiplexed in a single run:
Primers were ordered from Eurofins MWG Operon and HPSF (high purity salt-free) purified. PCRs were carried out in quadruplicate to decrease the possibility of random mispriming affecting the population of amplicons produced. No-template PCR controls were also included to exclude contamination.

PCR was performed in a 96 well plate, with each well containing a 25μl solution. Each 25μl reaction contained 1μL each of forward and reverse primers (10μM), 1μl of template (extracted) DNA, 0.5 μl of dNTP mix (10mM each), 2.5 μl of FastStart 10x Buffer, 0.25μl of 5U/μl FastStart HiFi Polymerase (Roche), 1μl of 20g/mL BSA (Sigma), and 6.5μl of 5M Betaine (Sigma), with the remainder of the 25μl being Molecular Biology Grade Water.

PCR reactions were assembled within a PCR hood in which all surfaces and equipment were decontaminated using ‘DNAase free’ solution (Ambion) and had been UV-irradiated for 20 minutes.

Thermal cycling was performed using a PTC-225 (MJ Research) thermal cycler. It consisted of initial denaturation at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 20 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 5 minutes, ending on a final hold at 4°C.

**Amplicon Pooling, Quantification and Pyrosequencing**

The quadruplicate amplicons were pooled together (total volume 100μl) and then purified twice (AMPure XP kit, Agencourt) following the recommendations in the Roche amplicon sequencing protocol and using a ratio of 0.7 volumes of AMPure beads to 1 volume of PCR product. The DNA content of the cleaned pool was quantified using the Quant iT PicoGreen dsDNA assay (Invitrogen) following the manufacturer’s instructions and then diluted in Tris-
EDTA to obtain a final pool containing $10^5/\mu L$ molecules. 30μl of this pool was added to the emulsion PCR reaction to attain a ratio of 0.3 molecules per bead. Sequencing of 16S rRNA amplicons was carried out on a Roche 454 FLX Titanium pyrosequencing platform by the Biochemistry Department’s Sequencing Facility at the University of Cambridge and processed using the shotgun processing pipeline. Raw data was returned as an SFF file to the Molecular Genetics and Genomics Group, NHLI, Imperial College, London.

**Preparation of Gene Sequencing Raw Data**

Reads were extracted per sample from the sequencing file by matching barcode sequences. The flowgrams for each sample were clustered, with poor quality reads being removed by AmpliconNoise\textsuperscript{126} running on Imperial College London’s Bioinformatics Support Service compute cluster. In addition chimeric sequences created during PCR were detected using Perseus\textsuperscript{126} and also removed. This curated set of reads, now separated by sample was used in all further analyses.

Curated reads were clustered into OTUs (operational taxonomic units) at 97% identity using UCLUST in the QIIME pipeline\textsuperscript{127}. The most abundant read in each OTU was selected as a representative sequence and this was compared to the SilvaNR version 111 database using a retrained RDP Classifier. OTUs are identified by the lowest taxonomic level of ID that was possible for the reference sequence and a unique OTU number, e.g. Pseudomonas_aeruginosa_321.

The numbers of reads per sample ranged from between 0 and 3502. Rarefaction (random re-sampling of reads per sample to obtain a representative set) was conducted in QIIME to 529 reads, retaining 94 samples of the original 111 sequenced and 209 OTUs. Procrustes analysis in QIIME was used to compare rarefaction at the next possible level (709 reads), but the
datasets were not significantly different, so 529, which retained an additional stable post treatment sample, was deemed acceptable.

**Statistical Methodology**

Three measures of diversity were utilised in this study.

Species richness is the number of bacterial species per sample, a simple measure of diversity that does not take into account the abundance of each bacterial species in a sample.

The Shannon Diversity Index is calculated by using the relative abundance ($x$) of each OTU in a sample. For each OTU the Shannon diversity index is determined first by using the equation:

$$-1 \times \left( \frac{x}{\ln(x)} \right)$$

Each OTU value in a sample is then summed together to give the Shannon diversity index for that sample. The Shannon diversity index was used as the measure of diversity (as opposed to other measures of diversity such as the Simpson index) as the logarithmic transformation performed reduces the weight of the abundant species versus the rarer species in the calculation, giving a more accurate representation of changes in a bacterial population which contains a dominant organism such as *Pseudomonas aeruginosa*.

The evenness of a sample represents how similar the relative abundance of each bacterium is in a sample. The Pielou’s evenness index ($J'$) of a sample with $S$ number of species, is determined from the Shannon diversity index ($H'$) using the equation:

$$J' = \frac{H'}{H'_{\text{max}}}$$

where
Each of these diversity indices give important information to understand community structure, with richness and evenness being more simple to understand measures and the Shannon diversity index allowing a calculation of diversity that includes both richness and evenness.

Continuous variables were compared using Mann-Whitney testing for non-paired data, and Wilcoxon Matched pairs test for paired data. Categorical variables were examined using Fisher’s exact test. Correlations were performed using Spearman’s rank correlation coefficient.

All data is expressed as median values with the interquartile range. All statistical analysis was performed using Graphpad Prism (GraphPad Software Inc).
5.3 Results

OTU Data

The number of OTUs ranged from 1 to 41 per sample. The commonest 20 OTU’s are listed below with their associated OTU identifier (Table 5.1). Some names appear more than once as identification of the OTU is not resolved to the species level. 27 samples were available for analysis of day 1 data. Of these, OTU 321 Pseudomonas aeruginosa, was the dominant OTU identified in 21 samples with 6 having other bacteria identified as the dominant OTU (2 subjects had Achromobacter, 2 Staphylococcus aureus, 1 Streptococcus sp. and 1 Haemophilus influenzae) One patient had no detectable OTU 321 Pseudomonas aeruginosa; this subject did, however, have a positive microbiology culture for PA (Subject 20, Figure 5.1). Figure 5.1 demonstrates graphically the varying relative abundance of bacteria on day 1 of treatment between subjects. For day 1 samples, the median relative abundance of the OTU 321 Pseudomonas aeruginosa was 80 % (IQR, 55-90). There was no significant correlation between relative abundance of this OTU on day 1 and FEV₁, FEV₁ %predicted or FEV₁ as a percentage of baseline.
<table>
<thead>
<tr>
<th>Rank</th>
<th>OTU</th>
<th>%</th>
<th>Rank</th>
<th>OTU</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>321_s_Pseudomonas_aeruginosa</td>
<td>57.3</td>
<td>11</td>
<td>11_g_Actinomyces</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>190_g_Streptococcus</td>
<td>8.9</td>
<td>12</td>
<td>74_g_Prevotella</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>36_g_Staphylococcus</td>
<td>8.2</td>
<td>13</td>
<td>284_g_Porphyromonas</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>218_g_Streptococcus</td>
<td>5.3</td>
<td>14</td>
<td>162_s_Prevotella scopos</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>132_s_Achromobacter_xylosoxidans</td>
<td>3.7</td>
<td>15</td>
<td>221_g_Rothia</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>121_g_Streptococcus</td>
<td>2.2</td>
<td>16</td>
<td>291_g_Gemella</td>
<td>0.4</td>
</tr>
<tr>
<td>7</td>
<td>166_g_Granulicatella</td>
<td>1.5</td>
<td>17</td>
<td>109_g_Neisseria</td>
<td>0.4</td>
</tr>
<tr>
<td>8</td>
<td>194_g_Haemophilus</td>
<td>1.5</td>
<td>18</td>
<td>294_g_Derxia</td>
<td>0.3</td>
</tr>
<tr>
<td>9</td>
<td>192_g_Lactobacillus</td>
<td>1.1</td>
<td>19</td>
<td>214_g_Stenotrophomonas</td>
<td>0.3</td>
</tr>
<tr>
<td>10</td>
<td>293_g_Veillonella</td>
<td>1.1</td>
<td>20</td>
<td>154_s_Uncultured_oribacterium</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 5.1 Relative Abundance of the top 20 OTU’s for all samples. OTUs are identified by number, followed by whether it was identified to a genus (g) or species (s) and then the identified bacterial species or genus.
Figure 5.1 Relative Abundance of OTU’s on day 1 of treatment for each subject colour coded by sequence identity. The top 20 are shown with lower frequency OTU’s collated as ‘Other OTU’s’
Principal Co-ordinate Analysis of OTU data

Principle co-ordinate analysis (PCoA) was performed on the rarefied OTU data using the weighted UniFrac distance to determine if there was any clustering of samples based on clinical variables. A number of clinical variables were examined. These included age, FEV₁ % predicted, patient ID, day of treatment, and beta-lactam antibiotic used. While the study was aimed at hypothesis generation, these were the variables that were expected to show a clustering effect. However, significant clustering was only seen with patient ID, $R^2 = 0.69$ $p = <0.001$. Analysis of clustering was performed using adonis in R. Figure 5.2 shows a selected number of subjects with each colour representing a different subject, highlighting the clustering by patient ID.

Figure 5.2  Principal co-ordinate analysis of weighted Unifrac distances of 4 patients (each assigned a separate colour). Each point is noted with patient ID and day of treatment. This plot identifies that most clustering is due to patient ID with a lesser effect of treatment causing increased variance.
No clustering was seen between the structure of the bacterial community and day of treatment, suggesting that antibiotic therapy had no significant effect on bacterial communities as a whole. Using a bi-plot and overlaying bacteria it could be seen that the position of samples tended to correspond with the position of PA with *Streptococci* and *Staphylococci* associated with those samples separated from PA in PC1 (figure 5.3).

![Bi-plot of PCoA of variance in weighted Unifrac distances overlaid with taxa data.](image)

**Figure 5.3** Bi-plot of PCoA of variance in weighted Unifrac distances overlaid with taxa data. The size of the sphere representing a taxon is proportional to the mean relative abundance of the taxon across all samples.

**Comparisons of 16S rRNA Gene Sequencing Data with Microbiology Culture Results**

An examination of the rarefied OTU data identified one subject who did not have identifiable 16s rRNA gene sequences attributable to PA. In the microbiology culture result, the subject was noted to have $1 \times 10^3$ cfu/ml of non-mucoid PA, which is the lowest limit of detection of bacteria in the assay. Examination of the raw OTU data however, showed that gene sequence
reads attributable to PA were detected in the sample but at a low level and hence discarded during the process of rarefaction.

During antibiotic treatment, five subjects appeared to clear PA from bacterial cultures. An examination of the non-rarefied data confirmed at least one read of sequence attributable to PA was seen in all samples except for one of the subjects, in a sample taken on the last day of antibiotic treatment.

**Is There a Relationship Between Diversity Indices and Lung Function Measures at the Start of Antibiotic Therapy?**

To determine a possible relationship, bacterial diversity indices were correlated with spirometry values obtained prior to commencing antibiotic therapy. There was no correlation between species richness and any measure of spirometry. There was a significant correlation between FEV₁ and Pielou’s evenness index but this was not seen for FEV₁ % predicted or FEV₁ as a % of baseline. No significant correlations were seen between the Shannon diversity index and lung function measure. While no significant correlations were seen between age and bacterial diversity on day 1 of treatment, there was a trend towards a negative correlation between age and both Pielou’s evenness index and the Shannon diversity index, i.e. evenness and diversity were decreased in older patients, as shown in Table 5.2.
<table>
<thead>
<tr>
<th>Species Richness</th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁</td>
<td>0.23</td>
<td>0.29</td>
</tr>
<tr>
<td>FEV₁ % predicted</td>
<td>0.15</td>
<td>0.48</td>
</tr>
<tr>
<td>FEV₁ % of baseline</td>
<td>-0.16</td>
<td>0.46</td>
</tr>
<tr>
<td>Age</td>
<td>-0.19</td>
<td>0.38</td>
</tr>
<tr>
<td>Pielou’s Evenness Index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁</td>
<td>0.51</td>
<td>0.01</td>
</tr>
<tr>
<td>FEV₁ % predicted</td>
<td>0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>FEV₁ % of baseline</td>
<td>-0.14</td>
<td>0.54</td>
</tr>
<tr>
<td>Age</td>
<td>-0.38</td>
<td>0.06</td>
</tr>
<tr>
<td>Shannon Diversity Index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁</td>
<td>0.38</td>
<td>0.08</td>
</tr>
<tr>
<td>FEV₁ % predicted</td>
<td>0.24</td>
<td>0.27</td>
</tr>
<tr>
<td>FEV₁ % of baseline</td>
<td>-0.21</td>
<td>0.33</td>
</tr>
<tr>
<td>Age</td>
<td>-0.39</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table 5.2 Correlations between bacterial diversity indices and spirometry values on day 1 of treatment. p values were determined using Spearman’s rank correlation co-efficient.

**Diversity Indices during Antibiotic Therapy**

No measure of diversity (species richness, Pielou’s evenness index, or Shannon diversity index) showed significant changes from the day 1 value at any of the subsequent follow-up samples as shown in Table 5.3 and figures 5.4-5.6.
<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Median (IQR)</th>
<th>p value (c.f day 1 value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species Richness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>28</td>
<td>15 (8,23)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>21</td>
<td>12 (8,23)</td>
<td>0.64</td>
</tr>
<tr>
<td>End of Rx</td>
<td>21</td>
<td>10 (6,18)</td>
<td>0.25</td>
</tr>
<tr>
<td>Stable post</td>
<td>10</td>
<td>15 (9,24)</td>
<td>0.72</td>
</tr>
<tr>
<td><strong>Pielou's Evenness Index</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>28</td>
<td>0.30 (0.14,0.53)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>21</td>
<td>0.42 (0.30,0.58)</td>
<td>0.12</td>
</tr>
<tr>
<td>End of Rx</td>
<td>21</td>
<td>0.36 (0.25,0.46)</td>
<td>0.83</td>
</tr>
<tr>
<td>Stable post</td>
<td>10</td>
<td>0.32 (0.25,0.54)</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>Shannon Diversity Index</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>28</td>
<td>0.77 (0.27,1.6)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>21</td>
<td>0.99 (0.65,1.7)</td>
<td>0.38</td>
</tr>
<tr>
<td>End of Rx</td>
<td>21</td>
<td>0.75 (0.55,1.4)</td>
<td>0.86</td>
</tr>
<tr>
<td>Stable post</td>
<td>10</td>
<td>0.89 (0.53,1.6)</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 5.3  
Diversity indices during antibiotic therapy and a comparison with day 1 of treatment values.
Figure 5.4  Species richness at each measured time point of the exacerbation. No significant differences are seen between time points. Line represents median, box represents interquartile range and whiskers are the minimum and maximum values.

Figure 5.5  Pielou’s evenness index at each measured time point of the exacerbation. No significant differences are seen between time points. Line represents median, box represents interquartile range and whiskers are the minimum and maximum values.
Figure 5.6  Shannon diversity index at each measured time point of the exacerbation. No significant differences are seen between time points. Line represents median, box represents interquartile range and whiskers are the minimum and maximum values.
Is Bacterial Diversity Significantly Different Between Treatment Outcome Groups?

The data was then examined by comparing groups of subjects by treatment outcomes, success versus failure. There were no significant differences between the treatment outcome groups for any of the diversity indices at any time point in the study as detailed in table 5.4 and illustrated in figures 5.7 – 5.9.

<table>
<thead>
<tr>
<th>Diversity Index</th>
<th>Day of Rx</th>
<th>Treatment Success</th>
<th>Treatment Failure</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species Richness</td>
<td>1</td>
<td>14 (7,26)</td>
<td>14 (8,19)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>19 (11,28)</td>
<td>10 (6,19)</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>End Rx</td>
<td>10 (7,18)</td>
<td>12 (6,17)</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Stable</td>
<td>10 (7,25)</td>
<td>10 (13,24)</td>
<td>0.52</td>
</tr>
<tr>
<td>Pielou’s Evenness Index</td>
<td>1</td>
<td>0.28 (0.13,0.50)</td>
<td>0.20 (0.12,0.57)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.43 (0.33,0.62)</td>
<td>0.36 (0.15,0.59)</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>End Rx</td>
<td>0.32 (0.25,0.56)</td>
<td>0.47 (0.24,0.66)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Stable</td>
<td>0.26 (0.21,0.53)</td>
<td>0.71, (0.32,0.71)</td>
<td>0.38</td>
</tr>
<tr>
<td>Shannon Diversity Index</td>
<td>1</td>
<td>0.67 (0.26,1.3)</td>
<td>0.56 (0.25,1.8)</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.3 (0.84,1.9)</td>
<td>0.78 (0.21,1.8)</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>End Rx</td>
<td>0.75 (0.55,1.1)</td>
<td>1.1 (0.53,1.8)</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>Stable</td>
<td>0.84 (0.41,1.6)</td>
<td>0.32 (0.32,0.71)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 5.4  Diversity indices at each measured time point comparing treatment outcome groups. No significant differences are seen between the groups. P value is calculated using Mann-Whitney u-test.
Figure 5.7 A comparison of species richness in treatment outcome groups at each measured time point of an exacerbation treatment. No significant differences were seen at any time point between treatment success (blue) and treatment failure (red). Data is presented as median values with interquartile ranges.

Figure 5.8 A comparison of Pielou’s evenness indices in treatment outcome groups at each measured time point of an exacerbation treatment. No significant differences were seen at any time point between treatment success (blue) and treatment failure (red). Data is presented as median values with interquartile ranges.
Figure 5.9 A comparison of Shannon diversity indices in treatment outcome groups at each measured time point of an exacerbation treatment. No significant differences were seen at any time point between treatment success (blue) and treatment failure (red). Data is presented as median values with interquartile ranges.
Is There a Relationship Between the Change in Lung Function and the Change in Bacterial Diversity?

Comparing the change in bacterial diversity indices between the beginning and end of treatment with changes in spirometry values over the same period revealed no significant correlations (Table 5.5)

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔSpecies Richness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in FEV₁(L)</td>
<td>0.05</td>
<td>0.85</td>
</tr>
<tr>
<td>% change in FEV₁</td>
<td>0.05</td>
<td>0.84</td>
</tr>
<tr>
<td>ΔPielou's Evenness Index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in FEV₁(L)</td>
<td>-0.21</td>
<td>0.40</td>
</tr>
<tr>
<td>% change in FEV₁</td>
<td>0.02</td>
<td>0.93</td>
</tr>
<tr>
<td>ΔShannon Diversity Index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change in FEV₁(L)</td>
<td>-0.11</td>
<td>0.64</td>
</tr>
<tr>
<td>% change in FEV₁</td>
<td>0.01</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Table 5.5 Correlations between the change in bacterial diversity indices and the change in spirometry over the treatment course. p values determined using Spearman’s rank correlation co-efficient.
Is There a Relationship Between Changes in PA Bacterial Density and Diversity Indices?

Examining paired data, there was no significant correlation between the change in species richness and the change in PA bacterial density. However a significant negative correlation was seen with both Pielou’s evenness index ($R= -0.53$, $p=0.04$) and the Shannon diversity index ($R=-0.54$, $p=0.03$). (Table 5.6 and figures 5.10 and 5.11)

<table>
<thead>
<tr>
<th>Change in PA bacterial density vs.</th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔSpecies Richness</td>
<td>-0.40</td>
<td>0.13</td>
</tr>
<tr>
<td>ΔPielou’s Evenness Index</td>
<td>-0.53</td>
<td>0.04</td>
</tr>
<tr>
<td>ΔShannon Diversity Index</td>
<td>-0.54</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Table 5.6 Correlations between the change in PA bacterial density and the change in bacterial diversity indices.

Figure 5.10 The correlation between change in PA bacterial density (cfu/ml) over the treatment course and the change in the Pielou’s evenness index. $r = -0.53$, $p = 0.04$, correlations determined using Spearman’s rank correlation co-efficient.
Figure 5.11 The correlation between change in PA bacterial density (cfu/ml) over the treatment course and the change in the Shannon diversity index. $r = -0.54$, $p = 0.03$, correlations determined using Spearman’s rank correlation co-efficient.

**Does Antibiotic Choice Affect Bacterial Diversity?**

Bacterial diversity indices were compared at each time point for patients receiving meropenem and ceftazidime. As detailed in table 5.7, species richness was significantly greater in the ceftazidime group at day 7 and the end of treatment. Pielou’s evenness values did not differ significantly at any time-point. When examining the Shannon diversity index there was a trend to a lower value in the meropenem group but this did not reach statistical significance (0.60 in the meropenem cohort versus 1.3 in the ceftazidime cohort, $p = 0.06$). The data is graphically represented in Figures 5.12-5.14.
### Table 5.7  
Bacterial diversity indices in cohorts divided by beta-lactam antibiotic received during treatment. p-values between the meropenem and ceftazidime groups were determined using Mann-Whitney tests.

<table>
<thead>
<tr>
<th>Diversity Index</th>
<th>Day of Rx</th>
<th>Meropenem</th>
<th>Ceftazidime</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species Richness</td>
<td>1</td>
<td>14 (8,19)</td>
<td>15 (6,24)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=11</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>11 (6,14)</td>
<td>19 (17,23)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=10</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>End Rx</td>
<td>7 (6,10)</td>
<td>18 (12,19)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stable</td>
<td>15 (9,26)</td>
<td>19 (9,25)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=6</td>
<td>n=3</td>
<td></td>
</tr>
<tr>
<td>Pielou’s Evenness Index</td>
<td>1</td>
<td>0.31 (0.15,0.55)</td>
<td>0.25 (0.11,0.50)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=11</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.38 (0.27,0.44)</td>
<td>0.44 (0.32,0.63)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=10</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>End Rx</td>
<td>0.29 (0.18,0.40)</td>
<td>0.43 (0.26,0.59)</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stable</td>
<td>0.42 (0.20,0.60)</td>
<td>0.26 (0.26,0.32)</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=6</td>
<td>n=3</td>
<td></td>
</tr>
<tr>
<td>Shannon Diversity Index</td>
<td>1</td>
<td>0.85 (0.28,1.7)</td>
<td>0.56 (0.25,1.2)</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=11</td>
<td>n=12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.84 (0.54,1.1)</td>
<td>1.3 (0.97,1.8)</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=10</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>End Rx</td>
<td>0.60 (0.31,0.74)</td>
<td>1.3 (0.67,1.6)</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stable</td>
<td>1.2 (0.4,1.9)</td>
<td>0.26 (0.26,0.32)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=6</td>
<td>n=3</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.12  
(a) Species richness during antibiotic therapy comparing those subjects receiving meropenem (red) with those receiving ceftazidime (blue). Species richness was significantly lower at day 7 and end of treatment in the meropenem cohort. Data is presented as median values with interquartile ranges.

(b) Species richness during antibiotic therapy comparing those subjects receiving meropenem (red) with those receiving ceftazidime (blue). Species richness was significantly lower at day 7 and end of treatment in the meropenem cohort. Data is presented as individual subjects.
Figure 5.13  Pielou’s evenness index during antibiotic therapy comparing those subjects receiving meropenem (red) with those receiving ceftazidime (blue). No significant differences were seen between cohorts. Data is presented as median values with interquartile ranges.

Figure 5.14  Shannon diversity index during antibiotic therapy comparing those subjects receiving meropenem (red) with those receiving ceftazidime (blue). Shannon diversity index trended to a lower value at the end of treatment in the meropenem cohort. Data is presented as median values with interquartile ranges.
Does The Presence of PA Hypermutators Affect Bacterial Diversity?

Bacterial diversity indices were compared between groups divided on the basis of isolation of PA hypermutator strains. No significant differences were seen for any of the calculated diversity indices at either the beginning of the study or at the end of the treatment, when comparing groups using a Mann-Whitney test as detailed in Table 5.8. Samples for hypermutator strain isolation were not taken on day 7 of treatment or at the stable visit post-treatment.

<table>
<thead>
<tr>
<th>Diversity Index</th>
<th>Day of Rx</th>
<th>Hypermutator absent</th>
<th>Hypermutator present</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species Richness</td>
<td>1</td>
<td>11 (7,16) n=11</td>
<td>22 (7,31) n=10</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>End of Rx</td>
<td>13 (7,19) n=6</td>
<td>11 (6,20) n=10</td>
<td>0.91</td>
</tr>
<tr>
<td>Pielou’s Evenness</td>
<td>1</td>
<td>0.20 (0.10,0.57) n=11</td>
<td>0.37 (0.13,0.54) n=10</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>End of Rx</td>
<td>0.32 (0.24,0.48) n=6</td>
<td>0.31 (0.19,0.48) n=10</td>
<td>1.0</td>
</tr>
<tr>
<td>Shannon Diversity</td>
<td>1</td>
<td>0.41 (0.25,1.50) n=11</td>
<td>1.20 (0.24, 1.90) n=10</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>End of Rx</td>
<td>0.83 (0.49,1.4) n=6</td>
<td>0.72 (0.43,1.50) n=10</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Table 5.8  Bacterial diversity indices in the presence and absence of PA hypermutator strains.

No significant differences were seen between groups at either time-point measured. p-values were determined using Mann-Whitney test.
Does Bacterial Diversity at the End of an Exacerbation Predict Time to Next Exacerbation?

Bacterial diversity indices at the end of antibiotic treatment were correlated with time to next exacerbation values to determine if a relationship existed. No significant correlations were seen with any of the indices calculated as detailed in Table 5.9.

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTNE vs. diversity index at end of Rx vs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species Richness</td>
<td>0.09</td>
<td>0.71</td>
</tr>
<tr>
<td>Pielou’s Evenness Index</td>
<td>-0.14</td>
<td>0.56</td>
</tr>
<tr>
<td>Shannon Diversity Index</td>
<td>-0.14</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Table 5.9 Correlations between bacterial diversity indices at the end of antibiotic therapy and time to next exacerbation values. No significant correlations were found. Correlations were performed using Spearman’s rank correlation co-efficient.
5.4 Discussion

This aim of this study was to determine whether bacterial communities in the CF lung changed significantly during antibiotic treatment of an infective exacerbation and if so, did this have an effect on treatment outcome. Previous studies have shown that antibiotic therapy for exacerbations does not significantly affect bacterial diversity\textsuperscript{29,31,129}. Some of these studies have utilised T-RFLP\textsuperscript{29,30}, a technique which has a much lower sensitivity for detecting individual species than 16S rRNA gene sequencing. Two studies have utilised 16s rRNA sequencing to examine exacerbations. Fodor et al.\textsuperscript{129} examined samples taken from 23 subjects during an exacerbation and demonstrated only a small decrease in species richness during antibiotic treatment. Zhao et al.\textsuperscript{31} noted a greater change during treatment but recovery of the bacterial community after treatment.

The data presented in this study would suggest that bacterial diversity is not significantly affected during treatment. Cluster analysis of the sequencing data revealed that samples clustered by subject ID more than any other variable; this suggests that each subject harbours a unique bacterial ecosystem. Equally, when examining co-plotting with bacteria, it was clear that PA was the dominant bacteria with most subjects clustering around this organism. The clustering around PA bacteria is perhaps to be expected given that the major inclusion criterion for the study was the presence of chronic PA infection. The results discussed here may therefore only relate to this patient group, although this is the majority of patients in the CF clinic.

Bacterial identification from 16S rRNA sequence data is based on known sequences from bacteria being entered into a database. The quality of the data is therefore very dependent on the quality of the information in the database. A possible example of mis-identification can be seen in the top 20 OTUs (Table 5.1). At number 18 is an OTU identified as Derxia sp.
This is a genus of organism found in tropical soils\textsuperscript{130} and lakewater\textsuperscript{131} and not noted to be a human pathogen. It does however have a close phylogenetic relationship with *Alcaligenes* \textit{sp.}\textsuperscript{132} which are more commonly seen in the CF lung. Contamination of the samples should also be considered although the methods have stringent processes to detect contamination. All sputum samples were handled using sterile equipment and all PCR reactions were performed within a PCR hood which had been decontaminated with ‘DNAse free’ solution and UV-irradiation. A no-template control was also added to the PCR reaction to maximise detection of contaminated materials.

When examining measures of bacterial diversity, namely species richness, Pielou’s evenness and the Shannon diversity index, it would appear that when all patients are analysed together there is no significant change in bacterial diversity during antibiotic treatment. Subgroup analysis allows further information to be gleaned from the data. One aim of this study was to determine if treatment outcome was related to changes in bacterial diversity. In the previous clinical tracking study it was demonstrated that the treatment failure group were more unwell at the start of treatment with a greater drop in FEV\textsubscript{1} from baseline. In this current study there were no significant differences in diversity measures between the treatment outcome groups at day 1 or any subsequent time-points. This may well be related to two factors. Firstly when spirometry measures were correlated with bacterial diversity measures, no relationship was seen with FEV\textsubscript{1} as a percentage of baseline. However there was a trend towards a relationship between Pielou’s evenness and the Shannon Diversity index with FEV\textsubscript{1} and, to a lesser degree, FEV\textsubscript{1} % predicted. This suggests that diversity is related more to the chronic baseline lung function measure of patients rather than the severity of the exacerbation (as determined by FEV\textsubscript{1} as a percentage of baseline). The negative correlation seen between age of the
patient and bacterial diversity on day 1 would further strengthen this theory. A previous study has also noted this association of decreasing bacterial diversity with age.

An examination of changes in PA bacterial density did suggest that there was a significant negative correlation between both Pielou’s evenness index and the Shannon diversity index. This result is perhaps to be expected for a measure of evenness; as antibiotics kill the dominant organism present in the sputum so the bacterial environment becomes more even. The negative correlation with the Shannon diversity index suggests that as PA bacterial numbers decrease other bacterial species become more prominent, making the bacterial microbiome more diverse. The data therefore may suggest that the antibiotics are killing more PA bacteria than other bacteria present.

An analysis of the effect of antibiotic choice on changes in bacterial diversity provides further information. The majority of patients received either meropenem or ceftazidime as the beta-lactam antibiotic choice. Ceftazidime is a cephalosporin antibiotic with anti-pseudomonal activity. It has good activity against Gram positive bacteria and *Pseudomonas aeruginosa* but lesser effects on anaerobic bacteria. Meropenem however is active against Gram positive and Gram negative bacteria as well as anaerobic bacteria. In this study there was a trend to lesser diversity at the end of treatment in those subjects receiving meropenem. The differences in bacterial diversity had resolved by the stable follow-up observation. It could be speculated that repeated courses of meropenem could decrease bacterial diversity at a greater rate than ceftazidime a finding that requires further longitudinal studies.

This study is limited by its design of recruiting patients at the time of exacerbation. A more detailed study would involve patients being recruited and providing clinical data and sputum samples when they are stable. This would allow detection of any changes in the microbiome in the lead up to an exacerbation.
In summary, this microbiota analysis in CF patients has confirmed that each patient’s microbiome is unique. Antibiotic treatment has been demonstrated to have no significant effect on the bacterial diversity within this microbiome. An association was seen between changes in bacterial numbers of PA and diversity indices suggesting PA is the major pathogen affected by antibiotic therapy. Subjects treated with meropenem had lower diversity measures at the end of therapy than those receiving ceftazidime. It remains to be seen in a longitudinal study whether repeated courses of meropenem decreases bacterial diversity more rapidly over time than ceftazidime. Low bacterial diversity is associated with lower lung function\textsuperscript{31,129}. The causal relationship between them however remains unclear. A longitudinal study with a large number of patients would be required to elucidate this relationship allowing analysis of patients with similar levels of diversity, stratified by the severity of lung function deficit.

If bacterial diversity is not changing significantly during exacerbations and antibiotic therapy perhaps a change is occurring in the expression of virulence factors within PA populations to trigger exacerbations? Quorum sensing systems, which can activate bacterial virulence factors, will be examined in the following chapter to determine if it plays a role in the pathogenesis of exacerbations.
CHAPTER 6

QUORUM SENSING BY PSEUDOMONAS AERUGINOSA

IN CF EXACERBATIONS

6.1 Introduction

A number of bacteria secrete quorum-sensing molecules as a form of communication between organisms. PA is probably one of the most studied of the bacterial communication systems. There are a number of quorum-sensing molecules produced by PA, falling into 2 main groups, the N-acyl-homoserine lactone group (which includes N-butanoyl-homoserine lactone (C4-AHL) and N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-AHL)) and the 4-hydroxy-2-alkylquinolones (HAQs) group (including 2-heptyl-3,4-dihydroxyquinolone (PQS), its biosynthetic precursor, 4-hydroxy-2-heptylquinolone (HHQ), and 2-heptyl-4-hydroxyquinolone N-oxide (HQNO)).

As previously discussed QS systems are involved in the activation of bacterial virulence factors including the production of rhamnolipids\(^\textsuperscript{136}\) and alkaline protease\(^\textsuperscript{137}\). No previous study has shown whether QS signalling is affected by antibiotic treatment and whether that has an effect on clinical response. By utilising techniques noted in previous studies\(^\textsuperscript{138}\), these QS molecules can be measured directly from sputum samples allowing an in-depth investigation of bacterial signalling during exacerbation therapy.

This study examined sputum levels of C4-AHL, 3-oxo-C12-AHL, HHQ, HQNO and PQS during treatment of an infective exacerbation of CF and at a stable level post treatment. These molecules were extracted from sputum samples and quantified using a process of liquid chromatography-tandem mass spectrometry (LC-MS/MS).
Aims of the study

1. To determine whether a relationship exists between QS molecule concentration and markers of severity of an exacerbation
2. To determine if QS molecule concentrations at the start of an exacerbation can predict treatment outcome responses.
3. To determine if mucoidy is associated with decreased QS molecule concentration
4. To determine if hypermutator strains are associated with lower decreased QS molecule concentration
5. To determine if QS levels change during antibiotic therapy and if this is related to changes in PA bacterial density.

6.2 Methods

The quorum sensing molecule assays were kindly performed by Nigel Halliday, School of Molecular Medicine, University of Nottingham from sputum samples collected during the clinical tracking study as described in Chapter 2.

Sample Preparation

Extracts of sputum samples for LC-MS/MS analysis were prepared by solvent extraction. Up to 1.0 ml of 50% (v/v) sputum suspension was spiked with 10 µl of an internal standard mix (1.0 µM solution of deuterated PQS (PQS-d₄) in MeOH), and extracted in triplicate with 0.5 ml volumes of 0.01% (v/v) AcOH in EtOAc. After the addition of acidified solvent the samples were vortexed for approximately 1 min. The two immiscible layers were encouraged to separate fully by centrifugation (3 min at 13000 rpm) with the analytes of interest partitioning into the organic phase. The combined organic extracts were dried under vacuum.
Dried extracted samples were re-solved in 50 µl of 0.1% (v/v) formic acid in MeOH prior to LC-MS/MS analysis.

**Preparation of Calibration and Quality Control (QC) Standards**

In the absence of blank sputum samples to spike with analytes and produce matrix matched calibration and QC samples, 1.0 ml aliquots of 0.9% (w/v) NaCl were used, spiking with 50 µl of methanolic analyte mix at 0, 5, 25, 50, 100, 200, 400, and 1000 nM. QC samples were prepared at 75 and 800 nM.

All calibration and QC samples were prepared in triplicate, extracted and prepared ready for LC-MS/MS analysis as described above.

**LC-MS/MS Analysis**

LC-MS/MS analysis was conducted on a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer in tandem with a Shimadzu series 10AD VP LC system as previously published\(^1\)\(^3\)\(^9\). 20 µl of the prepared sputum samples were injected into the LC instrument for analysis. The chromatographic separation was achieved using a Phenomenex Gemini C18 reversed phase column (3.0 µm, 100 x 3.0 mm) with a constant mobile phase flow rate of 450 µl/min. Mobile phases consisted of aqueous 0.1% (v/v) formic acid (A) and 0.1% (v/v) formic acid in MeOH (B). The binary gradient began initially at 10% B and ran isocratically for the first 1 min before increasing linearly to 50% B over 1.5 min. After a further linear increase to 99% B over 4 min, the gradient was maintained at this composition for 2 min. The gradient was then returned to 10% B over 0.2 min and allowed to re-equilibrate for 2.3 min. The MS, operating in the positive electrospray (+ES) mode, was set up for multiple reaction monitoring (MRM) to constantly screen the eluent from the LC column for all the analytes of interest.
**Sample Quantification**

For each analyte, ratios of LC-MS/MS peak areas to internal standard (d4-PQS) peak areas were calculated and used to construct calibration lines of peak area ratio against analyte concentration (see Appendix 3). Results from the QC samples were used to assess precision and accuracy at both the high end and low end of the calibration lines. Sample concentrations for each analyte (calculated from the slope of the calibration line) were then adjusted to reflect the differing volumes of sputa that each sample was prepared from, to give a final calculated sputum concentration.

**Statistical Methodology**

Continuous variables were compared using Mann-Whitney testing for non-paired data, and Wilcoxon Matched pairs test for paired data. Categorical variables were examined using Fisher’s exact test. Correlations were performed using Spearman’s rank correlation coefficient.

All data is expressed as median values with the interquartile range. All statistical analysis was performed using Graphpad Prism (GraphPad Software Inc).


6.3 Results

Day 1 QS Analyte Concentrations

28 subjects provided samples for the day 1 analysis. The samples for the subject excluded from the clinical study for not culturing PA was analysed and contained no measured QS analytes.

Four subjects had no detectable QS analytes at day 1. Of these, three had no detectable QS analytes for any time-point measured and one only had measurable QS analytes at the stable follow-up visit.

For each analyte, it was determined what proportion of subjects had no detectable level of the analyte at any time point. There were no significant differences in age of those subjects who had detectable QS analytes when compared with those who had no detectable analytes as detailed in Table 6.1. Table 6.2 and figure 6.1 represent the median concentrations of each analyte on day 1.

<table>
<thead>
<tr>
<th></th>
<th>No detectable QS analyte</th>
<th>Detectable QS analyte</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Age (years)</td>
<td>n</td>
</tr>
<tr>
<td>C4-AHL</td>
<td>16</td>
<td>30 (25,42)</td>
<td>12</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>14</td>
<td>30 (20,47)</td>
<td>14</td>
</tr>
<tr>
<td>HHQ</td>
<td>12</td>
<td>30 (21,46)</td>
<td>16</td>
</tr>
<tr>
<td>HQNO</td>
<td>3</td>
<td>30 (18,42)</td>
<td>25</td>
</tr>
<tr>
<td>PQS</td>
<td>14</td>
<td>35 (24,46)</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 6.1 Detection of QS analytes in samples at all time points and median ages
<table>
<thead>
<tr>
<th>Day 1 QS analyte concentration (nM)</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4-AHL</td>
<td>6 (0.96)</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>13 (1.2, 89)</td>
</tr>
<tr>
<td>HHQ</td>
<td>0 (0.91)</td>
</tr>
<tr>
<td>HQNO</td>
<td>6.6 (0.83)</td>
</tr>
<tr>
<td>PQS</td>
<td>0 (0.54)</td>
</tr>
</tbody>
</table>

Table 6.2 Median Day 1 concentrations (nM) of the QS analytes

Figure 6.1 Median concentrations of QS analytes on day 1 of the study. Data is presented as box and whisker plots with the boxes representing median values and interquartile ranges and whiskers representing maximum and minimum values.
Is Age a Factor in QS Analyte Concentrations?

Correlations were performed between age and QS analyte concentrations at the beginning of an exacerbation. A significant negative correlation was seen for HQNO, with correlations for the other analytes not meeting significance but with a trend towards a negative correlation as detailed in table 6.3.

<table>
<thead>
<tr>
<th>Age vs. Day 1 QS analyte concentration</th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4-AHL</td>
<td>-0.35</td>
<td>0.09</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>-0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>HHQ</td>
<td>-0.39</td>
<td>0.06</td>
</tr>
<tr>
<td>HQNO</td>
<td>-0.44</td>
<td>0.03</td>
</tr>
<tr>
<td>PQS</td>
<td>-0.41</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 6.3 Correlations between subject age and QS analyte concentrations on day 1 of the clinical study. Correlations were performed using Spearman’s rank correlation coefficient.

Is There a Relationship Between Day 1 Lung Function and QS Analyte Concentration?

Correlations were performed between spirometry measures and QS analyte concentrations on day 1 of the study. No significant correlations were seen between any of the QS analytes and FEV$_1$. There was a significant correlation between C4-AHL and FEV$_1$ % predicted, but this was not seen for the other analytes. When examining the correlation between FEV$_1$ as a percent of baseline there appeared to be a trend to a negative correlation, but this only reached statistical significance for HQNO as detailed in Table 6.4.
Is There a Relationship Between Inflammatory Markers and QS Analyte Concentration?

Correlations were performed comparing inflammatory markers (C-reactive protein (CRP), total white cell count and neutrophil count) with QS analyte concentrations on day 1 of treatment. No significant correlations were seen. However when correlating both HQNO and QS with total white cell count and neutrophil count there did appear to be a weak correlation but this failed to reach statistical significance as detailed in table 6.5.

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FEV&lt;sub&gt;1&lt;/sub&gt; vs. day 1 QS analyte concentration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0.33</td>
<td>0.12</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>0.14</td>
<td>0.52</td>
</tr>
<tr>
<td>HHQ</td>
<td>0.13</td>
<td>0.55</td>
</tr>
<tr>
<td>HQNO</td>
<td>0.13</td>
<td>0.55</td>
</tr>
<tr>
<td>PQS</td>
<td>0.00</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>FEV&lt;sub&gt;1&lt;/sub&gt;% pred vs. day 1 QS analyte concentration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0.43</td>
<td>0.04</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>0.08</td>
<td>0.71</td>
</tr>
<tr>
<td>HHQ</td>
<td>0.13</td>
<td>0.56</td>
</tr>
<tr>
<td>HQNO</td>
<td>0.12</td>
<td>0.57</td>
</tr>
<tr>
<td>PQS</td>
<td>0.05</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>FEV&lt;sub&gt;1&lt;/sub&gt;%OBL vs. day 1 QS analyte concentration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>-0.35</td>
<td>0.09</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>-0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>HHQ</td>
<td>-0.39</td>
<td>0.07</td>
</tr>
<tr>
<td>HQNO</td>
<td>-0.43</td>
<td>0.04</td>
</tr>
<tr>
<td>PQS</td>
<td>-0.39</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Table 6.4 Correlations between QS analyte concentrations and spirometry measures on day 1 of the clinical study. Correlations were performed using Spearman’s rank correlation coefficient.
Table 6.5  Correlations between QS analytes and inflammatory markers on day 1 of treatment.
Correlations were performed using Spearman’s rank correlation co-efficient.

<table>
<thead>
<tr>
<th>QS Analyte</th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP vs. day 1 QS analyte concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>-0.07</td>
<td>0.73</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>-0.02</td>
<td>0.91</td>
</tr>
<tr>
<td>HHQ</td>
<td>-0.05</td>
<td>0.80</td>
</tr>
<tr>
<td>HQNO</td>
<td>0.05</td>
<td>0.83</td>
</tr>
<tr>
<td>PQS</td>
<td>0.06</td>
<td>0.80</td>
</tr>
<tr>
<td>Total white cell count vs. day 1 QS analyte concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0.13</td>
<td>0.55</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>0.11</td>
<td>0.62</td>
</tr>
<tr>
<td>HQNO</td>
<td>0.29</td>
<td>0.16</td>
</tr>
<tr>
<td>HHQ</td>
<td>0.37</td>
<td>0.07</td>
</tr>
<tr>
<td>PQS</td>
<td>0.40</td>
<td>0.06</td>
</tr>
<tr>
<td>Neutrophil count vs. day 1 QS analyte concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0.12</td>
<td>0.19</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>0.19</td>
<td>0.37</td>
</tr>
<tr>
<td>HHQ</td>
<td>0.31</td>
<td>0.14</td>
</tr>
<tr>
<td>HQNO</td>
<td>0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>PQS</td>
<td>0.39</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Do QS Analyte Concentrations at Day 1 Predict Treatment Outcome?

Median QS analytes concentrations on day 1 of the study were compared between groups coholed by treatment outcome (success or failure as defined in the earlier clinical tracking study). No significant differences were seen between groups for any of the QS analytes as detailed in table 6.6. The same analysis was performed for the end of treatment values of QS analyte concentration. This again revealed no significant differences between treatment outcome groups for any of the analytes measured.
Table 6.6  QS analyte concentrations at the beginning and end of treatment comparing treatment outcome groups. Values are presented as medians with interquartile ranges.

<table>
<thead>
<tr>
<th>QS analyte</th>
<th>Rx failure</th>
<th>Rx success</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>n=7</td>
<td>n=19</td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0 (0,6.3)</td>
<td>0 (0,86)</td>
<td>0.28</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>0 (0,460)</td>
<td>6.6 (0,68)</td>
<td>0.76</td>
</tr>
<tr>
<td>HHQ</td>
<td>5.1 (0,42)</td>
<td>79 (0,151)</td>
<td>0.57</td>
</tr>
<tr>
<td>HQNO</td>
<td>17 (1.3,450)</td>
<td>9.7 (0,0,48)</td>
<td>0.43</td>
</tr>
<tr>
<td>POS</td>
<td>0 (0,33)</td>
<td>28 (0,150)</td>
<td>0.30</td>
</tr>
<tr>
<td>End of Rx</td>
<td>n=5</td>
<td>n=15</td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0</td>
<td>0 (0,23)</td>
<td>0.17</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>2 (0,130)</td>
<td>0 (0,2.8)</td>
<td>0.20</td>
</tr>
<tr>
<td>HHQ</td>
<td>0</td>
<td>0(0,17)</td>
<td>0.96</td>
</tr>
<tr>
<td>HQNO</td>
<td>5.1 (0,44)</td>
<td>0.70 (0,1,9)</td>
<td>0.27</td>
</tr>
<tr>
<td>PQS</td>
<td>0 (0,4,5)</td>
<td>0 (0,18)</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Do Mucoid Morphotypes of PA have Different Levels of QS Molecule Production?

The concentrations of QS analytes were measure on day 1 of treatment and grouped by whether the sputum sample had a positive culture of mucoid PA or not. Comparisons of these groups revealed the levels of HHQ and HQNO were significantly higher in sputum samples with mucoid PA present. This relationship was not seen for C4-AHL or 3-oxo-C12-AHL. There was a trend towards higher concentrations in the mucoid group for PQS but this failed to reach statistical significance as detailed in table 6.7 and figure 6.2.
<table>
<thead>
<tr>
<th>QS analyte</th>
<th>Non-mucoid</th>
<th>Mucoid</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>n=5</td>
<td>n=19</td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0 (0.24)</td>
<td>0 (0.11)</td>
<td>0.94</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>0 (0.410)</td>
<td>15 (0.92)</td>
<td>0.26</td>
</tr>
<tr>
<td>HHQ</td>
<td>0 (0.2.3)</td>
<td>27 (2.9,120)</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>HQNO</td>
<td>1.1 (0.1,7.4)</td>
<td>24 (5.7,190)</td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td>PQS</td>
<td>0 (0,3.3)</td>
<td>15 (0.79)</td>
<td><strong>0.09</strong></td>
</tr>
</tbody>
</table>

Table 6.7  Median QS analyte concentrations in sputum samples grouped by the presence or absence of mucoid PA. Data is expressed as median values with interquartile ranges.

Do Hypermutator Strains of PA Produce Greater Concentrations of QS Molecules?

Median concentrations of each QS analyte were determined for samples from day 1 of treatment for those samples where hypermutator strains were isolated and compared with those where the hypermutator strains were absent. No significant difference was seen between groups for any of the QS analytes measured, although a trend to higher concentrations of HHQ and HQNO in the hypermutator negative group was seen but this failed to reach statistical significance as detailed in table 6.8 and figure 6.3. Fisher’s exact tests comparing the number of subjects with hypermutator strains and the number of subjects without any significant QS analyte concentrations throughout treatment showed no statistical significant differences for any analyte.
<table>
<thead>
<tr>
<th>QS analyte</th>
<th>Hypermutator –ve</th>
<th>Hypermutator +ve</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 n=11 n=10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0 (0.63)</td>
<td>0 (0.8.2)</td>
<td>0.72</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>6.6 (0.200)</td>
<td>59 (0,130)</td>
<td>0.69</td>
</tr>
<tr>
<td>HHQ</td>
<td>88 (0,150)</td>
<td>3.8 (0,16)</td>
<td>0.08</td>
</tr>
<tr>
<td>HQNO</td>
<td>59 (1.4,450)</td>
<td>10 (0.6,30)</td>
<td>0.09</td>
</tr>
<tr>
<td>PQS</td>
<td>44 (0,150)</td>
<td>2.1 (0.19)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 6.8 Comparing QS analyte concentrations by the presence or absence of PA hypermutator strains. Data is expressed as median values with interquartile ranges.
Figure 6.2 Median QS analyte concentrations in sputum samples grouped by the presence or absence of mucoid PA. Data is expressed as box and whisker plots with the box representing median values and interquartile ranges and the whiskers representing minimum and maximum values. Significant differences are seen for the HHQ and HQNO analytes as noted.
Figure 6.3  Median QS analyte concentrations in sputum samples grouped by the presence or absence of hypermutator strains of PA. Data is expressed as box and whisker plots with the box representing median values and interquartile ranges and the whiskers representing minimum and maximum values. No significant differences are seen.
Do QS Analyte Levels Change During Antibiotic Therapy?

Each analyte was analysed separately. Any subject who had no measurable QS analyte throughout the study period was not included in the analysis of that analyte. This did not affect p-values as a paired sample t-test was performed. No significant difference was seen for levels of C4-AHL for subsequent time-points when compared with day 1 values. For all the other analytes there was a significant drop in QS analyte concentrations for each treatment time point as detailed in table 6.9 and figure 6.4. The stable post treatment values however were not significantly different to the day 1 values.

<table>
<thead>
<tr>
<th>QS Analyte</th>
<th>n</th>
<th>Median (IQR)</th>
<th>p value (compared with day 1 value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4-AHL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>13</td>
<td>7.4 (0.53)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>10</td>
<td>0 (0.0)</td>
<td>0.16</td>
</tr>
<tr>
<td>End of Rx</td>
<td>10</td>
<td>0 (0.13)</td>
<td>0.20</td>
</tr>
<tr>
<td>Stable post</td>
<td>6</td>
<td>0 (0.4.5)</td>
<td>0.25</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>15</td>
<td>68 (9,233)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>12</td>
<td>0 (0.30)</td>
<td>0.002</td>
</tr>
<tr>
<td>End of Rx</td>
<td>13</td>
<td>3.9 (0.59)</td>
<td>0.02</td>
</tr>
<tr>
<td>Stable post</td>
<td>5</td>
<td>7.2 (0.260)</td>
<td>0.37</td>
</tr>
<tr>
<td>HHQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>20</td>
<td>42 (5.6,137)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>17</td>
<td>0 (0.32)</td>
<td>0.02</td>
</tr>
<tr>
<td>End of Rx</td>
<td>18</td>
<td>0 (0.1,2)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Stable post</td>
<td>12</td>
<td>32 (7.6,75)</td>
<td>0.15</td>
</tr>
<tr>
<td>HQNO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>28</td>
<td>8.7 (0.35,57)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>24</td>
<td>0.3 (0.32)</td>
<td>0.03</td>
</tr>
<tr>
<td>End of Rx</td>
<td>24</td>
<td>0.9 (0.3,4)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Stable post</td>
<td>13</td>
<td>2.3 (0.3,48)</td>
<td>0.13</td>
</tr>
<tr>
<td>PQS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>16</td>
<td>33 (4,150)</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>14</td>
<td>6 (0.32)</td>
<td>0.02</td>
</tr>
<tr>
<td>End of Rx</td>
<td>14</td>
<td>0 (0.22)</td>
<td>0.001</td>
</tr>
<tr>
<td>Stable post</td>
<td>9</td>
<td>17 (0,107)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Table 6.9  QS analyte concentrations at each time point of the study. Data is presented as median values with interquartile range.
Figure 6.4  QS analyte concentrations during antibiotic therapy. Data is presented as a box and whisker plot with the box representing the median and interquartile ranges and the whiskers representing the minimum and maximum values. Significant p-values are documented.
Is QS Analyte Concentration Related to PA Bacterial Density?

Correlation was performed for QS analyte concentrations versus PA bacterial density on day 1 of the study. No significant correlations were seen for any of the measured analytes. Correlations were then performed between the changes in QS analyte concentration over the treatment course and the change in PA bacterial density. Again no significant relationship was found for any of the measured QS analytes as detailed in table 6.10

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 PA density vs. QS analyte concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>0.25</td>
<td>0.23</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>0.31</td>
<td>0.13</td>
</tr>
<tr>
<td>HHQ</td>
<td>0.16</td>
<td>0.45</td>
</tr>
<tr>
<td>HQNO</td>
<td>0.17</td>
<td>0.42</td>
</tr>
<tr>
<td>PQS</td>
<td>0.22</td>
<td>0.29</td>
</tr>
<tr>
<td>∆PA density vs. ∆QS analyte concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4-AHL</td>
<td>-0.22</td>
<td>0.36</td>
</tr>
<tr>
<td>3-oxo-C12-AHL</td>
<td>0.16</td>
<td>0.51</td>
</tr>
<tr>
<td>HHQ</td>
<td>0.26</td>
<td>0.29</td>
</tr>
<tr>
<td>HQNO</td>
<td>0.05</td>
<td>0.83</td>
</tr>
<tr>
<td>PQS</td>
<td>0.07</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table 6.10 Correlations between PA bacterial density and QS analyte concentration on day 1 of treatment and between the change in PA density and the change in QS analyte concentration over the course of antibiotic therapy.
6.4 Discussion

This study has determined that there may be a relationship between the sputum concentration of some QS molecules at the time of an exacerbation and the severity of the exacerbation as determined by percentage drop in spirometry. It has also determined that some QS molecule concentrations drop during antibiotic treatment which is not correlated with a change in bacterial numbers suggesting the inhibition of QS production by antibiotic therapy.

Is Age a Factor in QS Analyte Concentrations?

A previous study has suggested that PA quorum sensing systems are lost in late infection\textsuperscript{140}. In this study we did not examine time from first isolation of PA. However if age is used as a surrogate marker for this measure it has been shown that there were no significant differences in the median ages between those subjects who did produce QS molecules and those who did not. However, the median concentrations of the QS analytes on day 1 of treatment appeared to trend towards a negative correlation with age of the subjects. This only reached statistical significance for HQNO. This could represent a gradual decline in the number of bacteria within a chronic PA CF lung infection having a functioning QS system in keeping with the previous study. Previously it has been shown that mutations in the QS regulatory genes $lasR$ and $rhlR$ have been associated with a decrease in QS molecule production. In this current study, sputum was analysed directly without isolation of PA bacteria. To determine the mechanism of declining QS analyte concentration with age would require isolation of all distinct PA strains in a sample followed by sequencing of the QS regulatory genes to determine the incidence of mutation.
Is There a Relationship Between QS Analyte Concentrations and Lung Function Changes?

This is the first study to examine QS analyte levels in an acute exacerbation with directly obtained clinical sputum samples. It has been shown that QS analyte levels of HQNO and PQS are related to the severity of infection as determined by the negative correlations between these concentrations and FEV$_1$ as a percentage of the baseline. The results also demonstrate that QS analyte concentrations are not related to the other spirometry markers of FEV$_1$ and FEV$_1$ % predicted. It therefore would appear that QS molecule levels are related to the severity of the exacerbation for the individual subject rather than the absolute level of lung function. This is a very interesting observation and raises the possibility that the QS molecules themselves could be contributing to the severity of the exacerbation.

QS molecules have previously been shown to have a direct on CF airway epithelial cells. Mayer et al$^{41}$, have shown that 3-oxo-C12-AHL could stimulate CF epithelial cells to secrete IL-6 (IL-6 being a pro-inflammatory cytokine). The same QS molecule has been shown to induce other inflammatory mediators in human lung fibroblasts (although not a CF model) including IL-8, PGE-2 and cyclo-oxygenase 2 (Cox-2)$^{142;143}$. In the current study there were no significant correlations with serum inflammatory markers and QS analyte concentration although there was a trend to a positive correlation between both HQNO and PQS with total white cell count and neutrophil count on day 1 of treatment. However these are systemic inflammatory markers, a study of pulmonary inflammatory markers in sputum may show a more significant relationship.

Bacterial density has been shown to not alter significantly during the exacerbation and in this study no correlation was seen between PA bacterial density and QS analyte concentration for any of the molecules analysed. This suggests that at the time of exacerbation either the
relative abundance of QS-proficient PA organisms increase, or those QS-proficient PA organisms up-regulate their QS molecule production. Logically, the former is more likely. QS systems have presumably evolved with each bacteria secreting a similar quantity of QS molecules. If some bacteria increased their QS production, this would give a false representation of the number of bacteria present and trigger changes in virulence factors without the necessary numbers of bacteria to be effective.

To examine this further would require quantitative PCR of the lasR and rhlR genes, comparing stable sputum samples with those collected at exacerbation to determine the relative frequency of mutations in these QS regulating genes at each time point.

**Mucoidy and Quorum Sensing**

The relationship between PA mucoidy and QS analyte levels was also examine in this study. Previous studies have shown that mucoid PA produce greater quantities of C4-AHL than non-mucoid strains. In the current study, on day 1 of treatment there were no significant differences in C4-AHL or 3-oxo-C12-AHL median concentrations in sputum samples containing mucoid or non-mucoid samples. However, significantly higher concentrations of HHQ, HQNO and PQS were seen in sputum samples containing mucoid PA as compared with those only isolating non-mucoid PA. In the previous study, QS analytes were measured from PA cultures rather than clinical sputum samples, and the 4-hydroxy-2-alkylquinolones (HAQs) signalling systems were not measured. No previous work has identified this relationship between mucoidy and the HAQ signalling system. PQS has previously been shown to upregulate a number of virulence factors in PA including the production of rhamnolipids, iron chelation and the formation of biofilm in wild-type organisms. This finding of a relationship with mucoidy should be further examined with isolated bacterial cultures to confirm this relationship.
Hypermutator Strains of PA and Quorum Sensing

Hypermutator strains of PA have the ability to undergo rapid mutation to maintain their presence in chronic infections. As they undergo rapid mutation it could be postulated that they are more likely to develop mutations of the genes coding for the QS signalling systems and as such have decreased expression of QS molecules. A previous study\textsuperscript{140} has demonstrated increased mutation of QS regulatory genes in hypermutator strains of PA, although did not determine associated QS molecule concentrations. In this study, QS analyte concentrations in those samples containing hypermutator strains were not significantly different to those that did not. However there was a trend to lower levels of HHQ and HQNO in hypermutator-containing samples, although this did not reach statistical significance. It could be considered that hypermutator strains might eliminate all QS production and the data skewed by multiple zero values, but there was no significant difference in the number of subjects with absent QS production between groups cohorted by presence or absence of hypermutator strains. A significant limitation of this study was the hypermutator assay itself. The analysis of hypermutators was based only on their presence or absence in a sputum sample. Therefore in those sputum samples with hypermutators present, there would likely to have been significant numbers of non-hypermutator strains present. To determine whether hypermutator strains are having an effect on HHQ and HQNO production would require an assay based on known hypermutator strains grown in vitro.

Antibiotic Effect on Quorum Sensing

This study also examined the effect of antibiotic treatment on QS molecule levels. With the exception of C4-AHL, all QS analytes significantly decreased in concentrations during antibiotic treatment. The stable samples taken post-treatment were not significantly different to the day 1 levels taken prior to starting antibiotic therapy. It was also demonstrated that
changes in QS analyte concentrations were not related to the change in PA bacterial density. This suggests that the mechanism for a decrease in QS analyte concentration is either a selective killing of QS-proficient PA bacteria or an action of the antibiotic therapy to “switch off” the QS signalling system. It would be difficult to postulate why antibiotics would selectively kill QS-proficient bacteria, especially as quorum sensing is involved with upregulation of virulence factors to evade antimicrobial killing. Certainly it has been shown that a number of antibiotics can inhibit QS signalling. Skindersoe et al\textsuperscript{147} have previously demonstrated azithromycin, ceftazidime and ciprofloxacin could all inhibit QS signalling at sub-lethal dosing i.e. could inhibit quorum sensing without killing bacteria. Such “switching off” of QS signalling by antibiotic may explain why the QS analyte levels had increased again by the stable post-treatment measure. It has been previously shown that inhibiting quorum sensing in PA can increase biofilm eradication by antibiotics, make the biofilm more susceptible to neutrophil phagocytosis in murine models\textsuperscript{72} and eradicate biofilms in PA cultures\textsuperscript{148}. It is therefore possible that the effect of antibiotics in CF exacerbations is two-fold; it has an effect of inhibiting QS-molecule signalling which then increases the effect of its anti-microbial killing ability by weakening the biofilm and down-regulating virulence factors in the PA bacteria present. A limitation of this study was the widespread prophylactic use of azithromycin in the subject group, which was continued through the treatment. Twenty-three subjects were receiving azithromycin at the time of study. This may have affected QS molecule levels in some patients.

Effect of Antibiotic Therapy on QS regulation

The QS signalling system is both multi-system and hierarchical. As previously mentioned the AHL systems are regulated by the \textit{las} and \textit{rhl} genes; these systems are linked to the AQ system regulated through the \textit{pqs} genes. They act in a hierarchical fashion with the gene products of the \textit{las} system positively regulating the \textit{rhl} system (the \textit{las} system produces 3-
oxo-C12-AHL and the rhl system produces C4-AHL). The gene products of the las system also positively regulate the pqs system whereas the gene product of the rhl system are negative regulators of the pqs system (which produce HHQ, HQNO and PQS). There is also a feedback mechanism whereby pqs gene product inhibits the rhl system. To fully understand what effect antibiotic therapy has on this regulatory system and where it acts would require multiple assays of infection models with knock-out gene variants of PA bacteria. However the global decline in QS levels would suggest that antibiotic therapy is having an effect at the higher levels of the hierarchical system, possibly the las system. A further investigation of las mutants in a clinical infection model would be a priority for investigation.

The study described in Chapter 6 is the first study to measure quorum-sensing molecules produced by PA directly from sputum samples collected during an exacerbation. There appeared to be a relationship between the severity of an exacerbation, as determined by the FEV₁ on day 1 as a percentage of the stable baseline value, with levels of QS molecules (Table 6.4). Median concentrations of most QS molecules dropped significantly during treatment but had increased to pre-treatment levels by the stable follow-up time-point. The lack of association with changes in PA bacterial numbers suggested that the drop in concentrations represented an active process of inhibition of QS-molecule production. It has previously been demonstrated that QS production can be inhibited by sub-lethal concentrations of antibiotics and other compounds including garlic. QS molecules have also been shown to be pro-inflammatory. It is possible therefore that this QS inhibition effect of antibiotics may be involved in the recovery from exacerbation, both by reducing the inflammatory stimulus within the lung and by allowing more effective killing of bacteria as described in the previous chapter.
CHAPTER 7  
DISCUSSION AND PLANS FOR FUTURE STUDY

The thesis has provided an in-depth analysis of a number of aspects of the infective pulmonary exacerbation in cystic fibrosis. This work has shown agreement with previously published work in CF and has also provided new insights, particularly with respect to hypermutator strains of PA and quorum-sensing systems during antibiotic treatment of an infection.

The clinical tracking study was performed as a means of data collection for subsequent microbiological investigations but provides corroboration of previous observational and registry data analysis studies. Of the 27 subjects analysed in the clinical study, 8 failed to return to within 90 % of baseline lung function. Clearly the concern of failing to return to baseline is the cumulative effect of such exacerbations leading to inexorable decline in lung function with the associated significant morbidity and ultimately mortality. Analysis of the factors that lead to this failure revealed similar results of a far larger US Registry data analysis; those patients who failed to return to baseline had a bigger drop in lung function at the time of exacerbation and a longer time from stable measure to exacerbation. As discussed in Chapter 2, this may have significant implications for the treatment of CF patients. If this longer time between stable measures and exacerbation were to actually represent patients waiting longer to start treatment after the onset of symptoms of an exacerbation, it would suggest that patients should start treatment promptly at the onset of symptoms. Clearly this would require both significant patient education and hospital bed management changes. The data as it stands however is not conclusive on the matter. It would require a more in-depth study requiring a cohort of patients to record daily symptoms, perhaps in a similar manner to the East London COPD cohort that has been extensively investigated. This would allow a
far more accurate estimation of the timing of onset of exacerbation symptoms and, if combined with home spirometry measures, would provide a wealth of data on the evolution of an exacerbation.

A further noted observation in the clinical tracking study was that, in those patients who returned to baseline lung function during treatment, the majority of lung function improvement was seen within the first 7 days of treatment. This had also been noted in a previous retrospective review of data collected by the US CF Twin and Sibling Study. The authors of this study noted lung function improvement plateaued after 7-10 days of treatment. The appropriate duration of antibiotic therapy for CF exacerbations is a topical subject. Previous Cochrane reviews\textsuperscript{152} have highlighted the dearth of good-quality randomised studies investigating the problem. The lack of further improvement after 7 days of treatment raises a number of issues. For those patients who have returned to baseline lung function at day 7, should antibiotics be stopped and the patient discharged? The answer to this depends on whether lung function is the only significant variable to consider regarding treatment response. The clinical tracking study showed that inflammatory markers are often in the normal range and did not correlate with severity of an exacerbation. Symptom questionnaires are not validated for exacerbations. In the quantitative microbiology study presented in Chapter 3, there were no significant changes in PA density at any treatment time-point compared with the start of treatment and in this study no new antibiotic resistance emerged during the antibiotic treatments. The apparent inhibition of quorum sensing during antibiotic therapy noted in Chapter 6 is noted at day 7 (although this is the first time-point measured after commencing treatment). It is possible that the time to next exacerbation (TTNE) could be decreased by stopping antibiotics early. A previous study examining the factors present at the end of treatment that led to a shorter TTNE, demonstrated that lower FEV\textsubscript{1} and older age was associated with shorter exacerbation-free times\textsuperscript{153}. The same group also showed that
extending antibiotics beyond 14 days had no effect on improvement in clinical variables other than symptom score. It may be too simplistic to suggest that all patients receive an antibiotic course of \( x \) number of days, and a study requiring patients to be randomised to duration and then further stratified on lung function may require unfeasibly large numbers of subjects to adequately power the study. Perhaps it would be more useful to look at threshold targets of treatment, for example stopping antibiotics when \( \text{FEV}_1 \) reaches 90% of baseline.

This however raises the point of what to do with patients who don’t return to baseline within 7-10 days, as was the case in the treatment failure group. As previously mentioned, extending antibiotic course has been shown to have no effect on lung function markers. A study to determine if a change of antibiotics after 7 days has added benefit may be very informative and relatively simple to design.

Bacterial identification in routine clinical practice is based upon culture of sputum samples. In this thesis, both routine culture results were analysed as well as bacteria identified through 16S rRNA sequencing. The routine cultures are performed on selective media plates to grow those bacteria that are thought to be pathogenic and to inhibit the growth of those thought to be commensal. This provides information such as bacterial numbers and can lead on to antibiotic sensitivity testing, but gives less information on bacterial diversity. 16s rRNA gene sequencing can provide a wealth of information regarding what bacteria are present but is less helpful in determining what organisms are pathogenic. It also only identifies the species of bacteria and cannot provide information regarding virulence factor expression such as mucoidy.

The major inclusion criteria for the study included chronic infection with PA. This was cultured from all patients, except one who cultured only *Achromobacter xylosidans* and was excluded from the data analysis. Using the 16s rRNA gene sequencing however it was shown that in 6 of the 27 subjects analysed, PA was not the dominant organism (based on the
number of gene sequences attributable to each bacterium). The dominant organisms in the other patients were all common respiratory pathogens but treatment was not directed at these bacteria. However the notion of a dominant organism based on gene sequence copy numbers is rather artificial and cannot be directly extrapolated to bacterial numbers in the CF lung and equally the most prolific organism is not necessarily the most pathogenic to the CF patient.

In the microbiology study, PA bacterial numbers at the start of an exacerbation were not associated with any clinical measures of severity of the exacerbation although there was a trend towards a correlation between a decline in bacterial numbers and improvement in lung function (Table 3.4). This suggests PA bacterial killing may play some role in lung function recovery during antibiotic treatment. The change in PA bacterial density was also demonstrated to be correlated with the change in bacterial diversity, namely Pielou’s evenness and the Shannon diversity index. This suggests that PA bacteria are decreasing in numbers making the bacterial population more even, and the relative abundance of other bacteria increases to make the population more diverse.

Decreasing bacterial numbers were also shown to be associated with the emergence of *Candida albicans* in sputum cultures (Table 3.6). In this study, the emergence of *Candida* did not have an effect on clinical outcomes. Previous studies have suggested chronic *Candida albicans* is associated with increased exacerbation frequency and long term FEV₁ decline but the emergence during treatment has not previously been examined. While oro-pharyngeal contamination could lead to its emergence in sputum samples during treatment, the association with PA bacterial numbers does suggest the organism is within the lung, and is likely a mirror of what we see in the microbiome data; of a decrease in PA bacterial numbers allowing other organisms to increase in relative abundance.
PA hypermutator strains have been documented for a number of years in CF sputum. The study documented in Chapter 4 is the first clinical study to examine the effect of their presence during exacerbation treatment. The presence of hypermutator strain of PA was associated with less severe exacerbations although the numbers in the study were small, limiting the interpretation of the results. As a pilot study however it does suggest the need for larger scale studies. The presence of hypermutators had no effect on bacterial diversity. Bacterial diversity was shown to decrease with age and hypermutator strains were seen more frequently with increasing age. It could therefore be suggested that an association may have been predicted. A larger study would allow multiple regression analyses to remove age as a confounding factor and may give more information regarding a possible association.

In recent years, with the introduction of multiplex gene sequencing, a number of investigators have examined the CF airway microbiome both in chronic stable states and at the time of exacerbation. The overwhelming message from these studies has been that each CF patient’s lungs harbour a unique community of bacteria which may becomes less diverse over time with increasing age and progressive lung disease. Studies examining bacterial diversity during exacerbations have shown resilience of the community with minimal changes at the time of treatment. The study described in Chapter 5 would agree with these studies. The original hypothesis being examined was that increased bacterial diversity was associated with a better outcome in exacerbations but no evidence has been shown to confirm this theory. The observation of decreased bacterial diversity during treatment in those receiving meropenem when compared with those receiving ceftazidime is perhaps worth exploring in a further study. This would require a longitudinal study over many years to determine if repeated courses of meropenem lead to a more rapid decline in bacterial diversity. The clinical relevance of such a decline in bacterial diversity remains unclear.
The study described in Chapter 6 is the first study to measure quorum-sensing molecules produced by PA directly from sputum samples collected during an exacerbation. The study demonstrates an active inhibition of QS molecule production during antibiotic treatment. This is not the first study to show antibiotics have this effect on bacteria but it is the first to show it occurring during the treatment of an exacerbation. It raises a number of questions regarding both the pathogenesis of an infective exacerbation and the response to treatment. The apparent relationship between QS molecule concentration and FEV\textsubscript{1} as a percentage of baseline on day 1 of treatment, but not absolute FEV\textsubscript{1} or FEV\textsubscript{1} % predicted suggests that QS molecule concentration may be directly related to the severity of an exacerbation. This may suggest a greater density of QS-proficient PA bacteria is present at the time of exacerbation, and their selective growth may be the trigger for an exacerbation. Clearly this would not be the scenario for all exacerbations, especially considering some subjects produced none of the QS molecules analysed or only a proportion of them.

The appearances of the active inhibition of QS molecule production during antibiotic therapy suggests another role for antibiotics beyond that of bacterial killing. This study furthers the argument for the clinical trials of non-antibiotic QS inhibitors, which may offer alternative therapies to treat or prevent exacerbations.

A better understanding of pulmonary infections in CF is a key component to continuing the advances in CF survival figures that have been seen over the previous decades. The collection of studies presented in this thesis has added to the knowledge of the pathogenesis and treatment of infective exacerbations of CF but equally has identified areas for further study.
PLANS FOR FUTURE STUDY

1. The Development of a CF Patient Cohort

The East London COPD cohort has provided a large quantity of high-quality research into that condition. The development of a CF patient cohort including daily symptom diaries and regular home spirometry, combined with data already collected at clinics and annual reviews would provide a wealth of information. A multi-centre approach could recruit a larger number of patients and provide significant data in a relatively short time period. The data collected would give a more detailed view of the pathogenesis of an exacerbation as well as potentially allowing earlier treatment.

2. Antibiotic Duration Study

As discussed above, the logistics and number of patients required to adequately power a study to determine the optimum duration of antibiotics may be unfeasible and not particularly clinically relevant. Two alternative studies could be considered. One would involve stopping antibiotics when patients reach a threshold of FEV\textsubscript{1} recovery (for example, 90%). Clearly this would exclude some patients who don’t have a significant decline in FEV\textsubscript{1} at the time of exacerbation and a protocol would need to include a maximum duration of antibiotic therapy (and what to do with those subjects who don’t reach the threshold). Another possible study would involve randomising patients to either a 14 day course of one antibiotic combination or a 14 day course involving a switch in antibiotics after 7 days. This would determine if further lung function improvement is possible after 7-10 days of treatment.

3. Larger scale Hypermutator assay

The data presented in this thesis suggested that hypermutator strains were associated with less severe exacerbations. The small number of subjects in the study limited the validity of this
finding and a larger scale study is indicated. The hypermutator assay as described is rather labour-intensive with multiple cultures of PA required. An assay based on gene sequencing of mutations associated with hypermutation may be possible although a number of mutations can lead to the development of a hypermutator phenotype which may limit its use.

4. Long term microbiome study

The study presented in Chapter 5 adds to the evidence that the CF lung microbiome changes slowly over time. Previous studies have shown that the biggest driver of change in the microbiome is antibiotic courses. The study shows that meropenem is associated with a greater decline in diversity during antibiotic treatment. A retrospective study of the relationship between bacterial diversity and the number of days of meropenem versus ceftazidime could be completed rapidly (and could be done with the samples collected in this study). However, a prospective study with regular sputum samples would provide more detail of the shifts in bacterial populations.

5. Quorum-sensing Inhibitors

The study presented in Chapter 6 suggests a role of antibiotics in quorum-sensing inhibition. A number of non-antibiotic compounds have been shown to have similar inhibitory properties and may provide alternative therapeutic options. Some compounds such as garlic have already been studied but further work is indicated to determine the clinical effect of such inhibitors.
Reference List


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Sequeiros IM, Jarad N. Factors associated with a shorter time until the next pulmonary exacerbation in adult patients with cystic fibrosis. *Chron Respir Dis* 2012; 9(1):9-16.
APPENDIX

1. Cystic Fibrosis Questionnaire – Revised (CFQ-R) UK Adolescent and Adult version
2. Brompton CF Questionnaire
3. Quorum Sensing Study - Calibration lines of peak area ratio against analyte concentration.
Adolescents and Adults (Patients 14 Years Old and Older)

Cystic Fibrosis Questionnaire - Revised

Understanding the impact of your illness and treatments on your everyday life can help your healthcare team keep track of your health and adjust your treatments. For this reason, this questionnaire was specifically developed for people who have cystic fibrosis. Thank you for your willingness to complete this form.

Instructions: The following questions are about the current state of your health, as you perceive it. This information will allow us to better understand how you feel in your everyday life. Please answer all the questions. There are no right or wrong answers! If you are not sure how to answer, choose the response that seems closest to your situation.

Section I. Demographics

Please fill-in the information or tick the box indicating your answer.

A. What is your date of birth?
   Date __________/__________/_________
   Day Month Year

B. What is your gender?
   ☐ Male  ☐ Female

C. During the past two weeks, have you been on holiday or out of school or work for reasons NOT related to your health?
   ☐ Yes  ☐ No

D. What is your current marital status?
   ☐ Single/never married
   ☐ Married
   ☐ Widowed
   ☐ Divorced
   ☐ Separated
   ☐ Remarried
   ☐ With a partner

E. Which of the following best describes your racial background?
   ☐ White - UK
   ☐ White - other
   ☐ Indian/ Pakistani
   ☐ Chinese/ Asian
   ☐ African
   ☐ Caribbean
   ☐ Other [not represented above or people whose predominant origin cannot be determined/mixed race]
   ☐ Prefer not to answer this question

F. What is the highest level of education you have completed?
   ☐ Some secondary school or less
   ☐ GCSEs/ O-levels
   ☐ A/AS-levels
   ☐ Other higher education
   ☐ University degree
   ☐ Professional qualification or post-graduate study

G. Which of the following best describes your current work or school status?
   ☐ Attending school outside the home
   ☐ Taking educational courses at home
   ☐ Seeking work
   ☐ Working full or part time (either outside the home or at a home-based business)
   ☐ Full time homemaker
   ☐ Not attending school or working due to my health
   ☐ Not working for other reasons
Section II. Quality of Life

Please tick the box indicating your answer.

During the past two weeks, to what extent have you had difficulty:

1. Performing vigorous activities such as running or playing sports
2. Walking as fast as others
3. Carrying or lifting heavy things such as books, shopping, or school bags
4. Climbing one flight of stairs
5. Climbing stairs as fast as others

During the past two weeks, indicate how often:

6. You felt well
7. You felt worried
8. You felt useless
9. You felt tired
10. You felt full of energy
11. You felt exhausted
12. You felt sad

Please circle the number indicating your answer. Please choose only one answer for each question.

Thinking about the state of your health over the last two weeks:

13. To what extent do you have difficulty walking?
1. You can walk a long time without getting tired
2. You can walk a long time but you get tired
3. You cannot walk a long time because you get tired quickly
4. You avoid walking whenever possible because it’s too tiring for you

14. How do you feel about eating?
1. Just thinking about food makes you feel sick
2. You never enjoy eating
3. You are sometimes able to enjoy eating
4. You are always able to enjoy eating

15. To what extent do your treatments make your daily life more difficult?
1. Not at all
2. A little
3. Moderately
4. A lot
Cystic Fibrosis Questionnaire - Revised

Adolescents and Adults (Patients 14 Years Old and Older)

16. How much time do you currently spend each day on your treatments?
   1. A lot
   2. Some
   3. A little
   4. Not very much

17. How difficult is it for you to do your treatments (including medications) each day?
   1. Not at all
   2. A little
   3. Moderately
   4. Very

18. How do you think your health is now?
   1. Excellent
   2. Good
   3. Fair
   4. Poor

Please select a box indicating your answer.

Thinking about your health during the past two weeks, indicate the extent to which each sentence is true or false for you.

<table>
<thead>
<tr>
<th>Sentence</th>
<th>Very true</th>
<th>Somewhat true</th>
<th>Somewhat false</th>
<th>Very false</th>
</tr>
</thead>
<tbody>
<tr>
<td>I have trouble recovering after physical effort</td>
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<tr>
<td>I have to limit vigorous activities such as running or playing sports</td>
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<td>I have to force myself to eat</td>
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<tr>
<td>I have to stay at home more than I want to</td>
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<tr>
<td>I feel comfortable discussing my illness with others</td>
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<tr>
<td>I think I am too thin</td>
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<tr>
<td>I think I look different from others my age</td>
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<tr>
<td>I feel bad about my physical appearance</td>
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<tr>
<td>People are afraid that I may be contagious</td>
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<tr>
<td>I get together with my friends a lot</td>
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<tr>
<td>I think my coughing bothers others</td>
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<tr>
<td>I feel comfortable going out at night</td>
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<tr>
<td>I often feel lonely</td>
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<tr>
<td>I feel healthy</td>
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<tr>
<td>It is difficult to make plans for the future (for example, going to college, getting married, getting promoted at work, etc.)</td>
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<tr>
<td>I lead a normal life</td>
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</tbody>
</table>
Section III. School, Work, or Daily Activities

Questions 35 to 38 are about school, work, or other daily tasks.

35. To what extent did you have trouble keeping up with your schoolwork, professional work, or other daily activities during the past two weeks?
   1. You have had no trouble keeping up
   2. You have managed to keep up but it’s been difficult
   3. You have been behind
   4. You have not been able to do these activities at all

36. How often were you absent from school, work, or unable to complete daily activities during the last two weeks because of your illness or treatments?
   □ Always   □ Often   □ Sometimes   □ Never

37. How often does CF get in the way of meeting your school, work, or personal goals?
   □ Always   □ Often   □ Sometimes   □ Never

38. How often does CF interfere with getting out of the house to run errands such as shopping or going to the bank?
   □ Always   □ Often   □ Sometimes   □ Never

Section IV. Symptom Difficulties

Please select a box indicating your answer.

Indicate how you have been feeling during the past two weeks.

39. Have you had trouble gaining weight? ........................................... □ □ □ □

40. Have you been congested? ......................................................... □ □ □ □

41. Have you been coughing during the day? ..................................... □ □ □ □

42. Have you had to cough up mucus? .............................................. □ □ □ □

43. Has your mucus been mostly: □ Clear □ Clear to yellow □ Yellowish-green □ Green with traces of blood □ Don't know
   How often during the past two weeks:
   □ Always   □ Often   □ Sometimes   □ Never

44. Have you been wheezing? ............................................................. □ □ □ □

45. Have you had trouble breathing? ................................................ □ □ □ □

46. Have you woken up during the night because you were coughing? ....... □ □ □ □

47. Have you had problems with wind? .............................................. □ □ □ □

48. Have you had diarrhea? ............................................................... □ □ □ □

49. Have you had abdominal pain? ................................................... □ □ □ □

50. Have you had eating problems? ..................................................... □ □ □ □

Please make sure you have answered all the questions.

THANK YOU FOR YOUR COOPERATION!
BCFQ Health Status Questionnaire

This questionnaire is designed to find out how CF symptoms affect you these days. Understanding the impact of your illness and treatment can help your doctor to plan your care and adjust any treatment that you may be receiving.

Instructions:
Please read through each listed question carefully and tick (✔) either True or False as it applies to you.

Please answer all the questions. If you think that the question is not relevant to you, tick false. If you are sure a question applies to you, even only some of the time, please tick true.

Thank you for your time in completing this questionnaire.

Questions about how your daily activities may be affected by CF symptoms. Please tick either True or False as it applies to you.

1. I have difficulty lifting heavy items................................................. True False
2. My breathing limits my choice of sporting activities ....................... True False
3. Climbing stairs takes extra effort...................................................... True False
4. I am out of breath when carrying shopping....................................... True False
5. My breathing causes me to take longer over housework..................... True False
6. Cough interrupts my daily activities.................................................. True False

Questions about CF treatment. Please tick either True or False as it applies to you. If you think that the question is not relevant to you, tick False.

7. I am spending an excessive amount of time doing chest physiotherapy... True False
8. I feel that my daily treatment is time consuming............................... True False

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9. Due to CF, I am having difficulty reaching a balance between life and treatment. ☐ ☐

**CF-38 Health Status Questionnaire**

10. I am concerned about the amount of time spent in hospital as an in-patient. ☐ ☐

These are questions related to CF symptoms. Please tick true or false as it applies to you. If you think that the question is not relevant to you, tick False.

11. I get indigestion. ☐ ☐

12. I have pain in my joints. ☐ ☐

13. Cough interrupts my sleep. ☐ ☐

14. I am suffering from headaches. ☐ ☐

15. I get headaches from excessive coughing. ☐ ☐

16. I feel nauseas from excessive coughing. ☐ ☐

17. I sometimes vomit from excessive coughing. ☐ ☐

18. I feel embarrassed by coughing in public (e.g. in restaurant or public places). ☐ ☐

19. I tend to suppress my cough when in public (e.g. in restaurant or public transport). ☐ ☐

20. Coughing often causes discomfort in my chest. ☐ ☐

21. I have difficulty concentrating. ☐ ☐

22. Due to the unpredictability of my bowel movement, I tend to go to places where public toilets are near by. ☐ ☐

The following questions relate to how you feel because of your CF.

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Please tick either True or False as it applies to you. If you think that the question is not relevant to you, tick False.

23. At times, I feel I am a burden to others. ......................................................... True  False

24. I feel anxious about becoming isolated from my friends and family. ......................................................... True  False

25. I am afraid of being rejected by others. ......................................................... True  False

26. My CF makes me think about death. ......................................................... True  False

These questions are about how your relationship with others may be affected by CF. Please tick either True or False as it applies to you. If you think that the question is not relevant to you, tick False.

27. I have difficulty establishing sexual or close relationships. ......................................................... True  False

28. I have difficulty maintaining close relationships. ......................................................... True  False

29. I feel guilty about the sacrifices my parents or partner may make for me. ......................................................... True  False

30. I feel guilty because my parents or partner are anxious over my illness. ......................................................... True  False

Because of your CF, have you any of the following concerns for the future? Please tick either True or False as it applies to you. If you think that the question is not relevant to you, tick False.

31. I worry about the possibility of needing a transplant. ......................................................... True  False

32. I feel I am unable to make long term plans. ......................................................... True  False

33. I expect my health to get worse. ......................................................... True  False

Some questions that relate to your weight and body image. Please tick either True or False as it applies to you. If you think that the question is not relevant to you, tick False.

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3. Quorum Sensing Study - Calibration lines of peak area ratio against analyte concentration

**C4-AHL Calibration 130209**

\[ y = 0.000146x \]
\[ R^2 = 0.987098 \]

**Oxo-C12-AHL Calibration 130209**

\[ y = 0.000034x \]
\[ R^2 = 0.996740 \]
HHQ Calibration 130209

\[ y = 0.000063x \]
\[ R^2 = 0.992483 \]

Sample Concentration / nM
Mean Peak Area / IS Ratio (n=3)

HQNO Calibration 130209

\[ y = 0.002661x \]
\[ R^2 = 0.991919 \]

Sample Concentration / nM
Mean Peak Area / IS Ratio (n=3)

PQS Calibration 130209

\[ y = 0.000082x \]
\[ R^2 = 0.999036 \]

Sample Concentration / nM
Mean Peak Area / IS Ratio (n=3)