UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Longitudinal Assessment of Cystic Fibrosis Pulmonary Disease using Clinical, Biochemical and Emerging Microbiological Techniques

by

Thomas William Vaisey Daniels

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ABSTRACT

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LONGITUDINAL ASSESSMENT OF CYSTIC FIBROSIS PULMONARY DISEASE USING CLINICAL, BIOCHEMICAL AND EMERGING MICROBIOLOGICAL TECHNIQUES

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Cystic Fibrosis (CF) causes chronic lower respiratory tract infection leading to morbidity and mortality. CF Pulmonary Exacerbations (CFPEs) cause accentuated symptoms and increase mortality. The definition and aetiology of CFPEs has however proved elusive. Recently, culture independent techniques have shown that there is much greater diversity of bacteria than previously detected by culture dependent methods. Building on this, in the work presented here, the bacteria in respiratory samples from adults with CF were studied over a 12 month period. Each subject provided thrice weekly sputum samples for analysis by culture and culture independent microbiological methods. Concurrently an in-depth assessment of their subjective and objective health using Visual Analogue Scales (VAS) and spirometry (FEV1) was undertaken. Inflammation markers were also measured.

A total of 2061 samples from fourteen adults (mean age 30.2; mean FEV1% predicted 53.3%; 6 females; 8 ΔF508 homozygotes) were collected. Subjective VAS measures correlated with objective spirometric measures. However, previously unsuspected complexity of subjective symptomatology was found. Ribosomal clone sequence analysis identified 90 different species, including 15 not previously reported in CF lung disease. Notably 44% of species detected were obligate anaerobes, and 72% were species previously associated with the human oro-pharynx. During the study period, subjects experienced 42 CFPEs requiring treatment. New species were not seen to enter the bacterial community as aetiological agents for CFPEs. However, whilst treatment for CFPEs caused a large fall in the proportion of anaerobic species, no significant change in the proportion of *Pseudomonas aeruginosa* was detected.

Significant and potentially important differences in bacterial community composition, structure and stability between subjects separated by gender, genotype and lung function were observed. Moreover, the presence of certain species correlated with subjects suffering frequent CFPEs.

The results presented here give new insights in to the complexity of symptoms and bacterial diversity in CF pulmonary disease.

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DECLARATION OF AUTHORSHIP

I, …THOMAS WILLIAM VAISEY DANIELS

declare that the thesis entitled:

LONGITUDINAL ASSESSMENT OF CYSTIC FIBROSIS PULMONARY DISEASE USING CLINICAL, BIOCHEMICAL AND EMERGING MICROBIOLOGICAL TECHNIQUES

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission

Signed: ……..

Date:…….

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Abbreviations

ABPA –	Allergic Bronchopulmonary Aspergillosis
ASL –	Airway Surface Liquid
BAL –	Broncho-Alveolar Lavage
Bcc –	*Burkholderia cepacia* Complex
BMI –	Body mass index
BSA -	Bovine Serum Albumin
cDNA -	complimentory DNA
CF –	Cystic Fibrosis
CFF –	Cystic Fibrosis Foundation
CFPE –	Cystic Fibrosis Pulmonary exacerbation
CFRD –	CF Related Diabetes
cfu –	colony forming units
Cif –	CFTR inhibitory protein
Cl^- -	Chloride
Cm -	Centimetre
CMF –	Clinical Monitoring Form
COPD –	Chronic Obstructive Pulmonary Disease
DEPC -	Diethyl Pyrocarbonate
DGGE –	Deanturing Gradient Gel Electrophoresis
DMSO –	Dimethylsulphoxide
DNA –	Deoxyribo-Nucleic Acid
dNTP -	deoxynucleotide triphosphate
DTT –	dithiothreitol
ECP –	eosinophilic Cationic Protien
EDN –	Eosinophil Derived Neurotoxin
ELISA –	Enzyme linked immunosorbanta assay
ENaC –	Epithelial Sodium (Na) Channel
ETT –	Endotracheal Tube
FEV1 –	Forced expiratory Volume in One Second
FEV1% predicted –	FEV1 as a percentage of the predicted value using European Coal Steel Standards
FISH –	Fluorescent *In Situ* Hybridisation
FVC –	Forced Vital Capacity
g -	g force
g -	gram
G+C –	Guanine and Cytosine
GES -	Guanidinium thiocyanate-EDTA-Sarkosyl
GWB -	General Wellbeing
H_2O_2 -	Hydrogen peroxide
HPA –	Helath Protection Agency
HRP –	Horse radish Peroxidase
huIL-8 –	Human IL-8
Hz -	Hertz
IL-8 –	Interleukin 8
IQR -	Interquartile Range
M -	Molar
MCBT –	Multiple Combination Bacteriocidal antibiotic Testing
MCID –	Minimal Clinically Important Difference

MLST -	Multi-Locus Sequence Typing
mg -	milligram
MIC_{90} –	Miniumum Inhibitory Concentration required to inhibit 90% of isolates
mL -	millilitre
mM –	milli Molar
MRSA –	Methycillin Resistant *Staphylococcus aureus*
MSSA –	Methycillin Sensitive *Staphylococcus aureus*
NA –	Not applicable
Na^{+} -	Sodium
NaCl –	Sodium Chloride
NE –	Neutrophil elastase
ng -	nanograms
Nm –	nano Molar
nm -	nanometres
NAA –	Nucleic Acid Extraction
OF -	oral flora
OR –	odds ratio
OR –	Odds ratio
PBS –	phosphate buffered saline
PCL –	periciliary Layer
PCR –	Polymerase Chain reaction
PMA –	Propidium monoazide
PRO –	Patient Reported Outcome
QoL –	Quality of Life
RCT –	Randomised Controlled Trials
RFLP –	Restriction Fragment Length Polymorphism
RPP -	Response Profile Plot
SaO2 -	Transcutaneous estimated arterial haemoglobin Oxygen Saturation
SCOTIE –	Sputum Changes over Time in Cystic Fibrosis
SCV –	Small Colony Variant
SD –	Standard deviation
SGH –	Southampton General Hospital
SMG –	*Streptococcus milleri* group
SOP -	Standard Operating Procedure
TMB -	3,3',5,5'-Tetramethylbenzadine
T-RF –	Terminal Restriction Fragment
T-RFLP -	Terminal Restriction Fragment Length Polymorphism
UK –	United Kingdom
USA –	United States of America
VAP –	Ventilator Associated Pneumonia
VAS -	Visual Analogue Scale
VFA –	Volatile Fatty Acids
WHO –	World Health Organisation
Zn -	Zinc

Chapter 1 - Introduction

Cystic Fibrosis (CF) is the most common life threatening recessively inherited disease affecting Caucasians. In the United Kingdom (UK) it affects approximately 1 in every 2500 live births (Collins 1992), and there are estimated to be approximately 8200 registered UK CF patients (Dodge *et al.* 2006). CF is a multi-system disease affecting the respiratory tract, liver, pancreas, gastro-intestinal tract, musculo-skeletal system and reproductive organs. The genetic defect causes failure of airway defences, resulting in chronic bacterial infection of the lungs (Boucher 2007; Konstan & Berger 1997). As a consequence the majority of deaths are due to respiratory failure or sepsis from repeated lung infections (Penketh *et al.* 1987). Survival has increased dramatically since the first accurate description of the condition by Andersen in 1938 (Anderson 1938). Currently the median predicted survival in the UK is 38.8 years (Cystic Fibrosis Trust 2009b) with reports predicting a continued increase in age of death over the coming years (Dodge *et al.* 2006; Elborn, Shale, & Britton 1991).

There is a pressing need to understand the complex microbiology of the CF airway. Despite improved treatment of pulmonary infections (Knowles & Boucher 2002; Ramsey *et al.* 1999; Retsch-Bogart *et al.* 2009), pulmonary infections still result in a reduction in quantity and impaired quality of life (QoL). Thus a better understanding of the interaction between microbes and the human lung may then lead on to new or improved treatment strategies, which may in turn lead to an improved life expectancy for people born with CF.

1.1 CF Pulmonary Exacerbations

1.1.1 Clinical Significance

CF Pulmonary Exacerbations (CFPEs) are important healthcare events for individual patients, who can suffer from increased dyspnoea, increased sputum production, fever, pain, loss of appetite, or absenteeism from school or work. Attempts to define CFPEs in more detail are outlined in section 1.1.3 below. They also have significant a significant effect on mortality (Liou *et al.* 2001). Corey *et al.* in 1997 established that a lower FEV1 and a faster rate of decline in FEV1 was associated with a greater mortality rate (Corey *et al.* 1997). Block *et al.* demonstrated, in a cohort of CF patients infected with multi-resistant bacterial organisms, that those with lower lung functions had more frequent CFPEs (Block *et al.* 2006).

Britto *et al.* demonstrated a marked negative impact of CFPEs on self-reported Quality of Life (QoL) using a number of validated age-specific health related QoL tools (Britto *et al.* 2002). This negative impact was independent of lung function, nutritional status and demographic features. On 0-100 point scales, with higher values representing greater well being, each CFPE was associated with a fall of 6 points in the physical well being score, and a 3 point fall in the psycho-social score. Interestingly the negative effects of CFPEs on health related QoL were measurable 6 months after the last event. The researchers were unable to comment on the mechanism behind the effect, nor on whether different severities of exacerbations, or treatment modalities (e.g. home vs. hospital treatment) affected the relationship. In a later study, the same group went on to demonstrate that treatment for CFPE in hospital was associated with improvement in health related QoL, whereas treatment at home was associated

with deterioration (Yi *et al.* 2004). The study had significant flaws, particularly that the two groups (inpatient treatment and outpatient treatment) were not well matched for age and that the tools used to assess health related QoL differed in the two age groups.

In addition retrospective studies assessed physiological outcome measures in well matched episodes of home and hospital treatment for CFPE in children (Nazer *et al.* 2006). In both groups significant improvements were demonstrated in spirometric outcomes, weight gain and transcutaneous arterial oxygen saturations ($Sa0_2$). Significant differences were shown in favour of hospital treatment, with greater improvements in FEV1 and shorter duration of antibiotic therapy. However, these studies can be criticised for being based on retrospective analysis and have not controlled for selection bias. One such bias would be that clinicians would favour hospital therapy for patients who were deemed, in some way, to be more ill. These patients would therefore have to gain from any treatment, thus falsely demonstrating greater efficacy for hospital vs. home treatment.

1.1.2 Costs

CFPEs have a significant health care related cost, although the costs that are specific to CFPEs as differentiated from other CF care costs are difficult to separate. In 1999 Lieu *et al.* (1999) calculated that mean costs per CF patient aged $\geq$18 was $13300 per annum, and that 47% of costs were attributable to hospitalisations, which for the purposes of this discussion can assumed to be for treatment for CFPEs. Disease severity also increased costs. Those with the most severe lung function (FEV1 $\leq$ 40 %) had mean costs of $43300 per annum. Since this study, a number of expensive therapies, including inhaled

DNAse (Fuchs *et al.* 1994), inhaled tobramycin (Ramsey *et al.* 1999) and oral azithromycin have become part of routine therapy. The full impact of these new therapies on costs of care for CFPEs is not known, although recent reviews have suggested overall reductions in economic costs are achieved with nebulised tobramycin and oral azithromycin (Equi *et al.* 2002; Saiman *et al.* 2003; Wolter *et al.* 2002).

1.1.3 Definition

Despite the clear importance of CFPEs to mortality, QoL, and health costs, as well as an outcome measure in a number of pivotal clinical trials, there is no standardised definition. Dakin *et al.* (2001) demonstrated that there was marked heterogeneity between CF clinicians in Australia in the value they placed in a wide variety of clinical and laboratory characteristics in the diagnosis of a CFPE. As an example of the lack of consensus between clinicians, the usefulness of only 50% of the variables offered could be agreed upon by 3 out of 4 respondents or more. In many observational clinical trials it has been common to use the decision to start antibiotic therapy as a surrogate for CFPEs (Mayer-Hamblett *et al.* 2007). For a number of reasons however this is sub-optimal. First of all, Johnson *et al.* have shown marked variations between different centres in rates of treatment for CFPE (Johnson *et al.* 2003). These differences are associated with differences in survival rates and FEV1. Most centres would advocate an 'as required' treatment strategy for CFPEs. A few, notable the Danish national centre in Copenhagen, practice a more aggressive strategy of regular three-monthly courses of intravenous antibiotics, irrespective of symptoms (Frederiksen *et al.* 1996), making comparisons based on hospital interventions as a definition of CFPE challenging. Furthermore the decision to

diagnose a CFPE and to start treatment is not always based of clinical measures alone. To a greater or lesser degree pulmonary symptoms are frequently present in adult CF patients (Hodson & Geddes 2007). Although there may be a discrete event, such as haemoptysis, which precipitates a diagnosis of CFPE, more often there is no such discrete event, and symptoms are part of a continuum. Thus, the point at which an increase in a pre-existing symptom reaches a threshold at which a CFPE is diagnosed may be viewed as a somewhat arbitrary one. These problems were recognised in 1994 in a Cystic Fibrosis Foundation consensus statement. Ramsey *et al.* (1994) recommended that a definition of a CFPE "should be established as soon as possible and universally used in clinical trials". They went on to recommend that "this definition should include clinical symptoms but not therapeutic interventions such as hospitalisation". Despite 16 years having passed since this call was made, and further calls since (Marshall 2004) there is still no accepted universal definition of a CFPE.

A CFPE has been described as 'an acute change in pulmonary symptoms related to an increase in airway secretions' (Goss & Burns 2007). However, numerous other definitions have been used, each attempting to provide a more robust and specific definition. In particular, in the 1990s two major interventional randomised controlled trials laid out diagnostic criteria for the diagnosis of CFPEs (Fuchs *et al.* 1994; Ramsey *et al.* 1999). The definitions used in each are laid out in Figure 1-1 below.

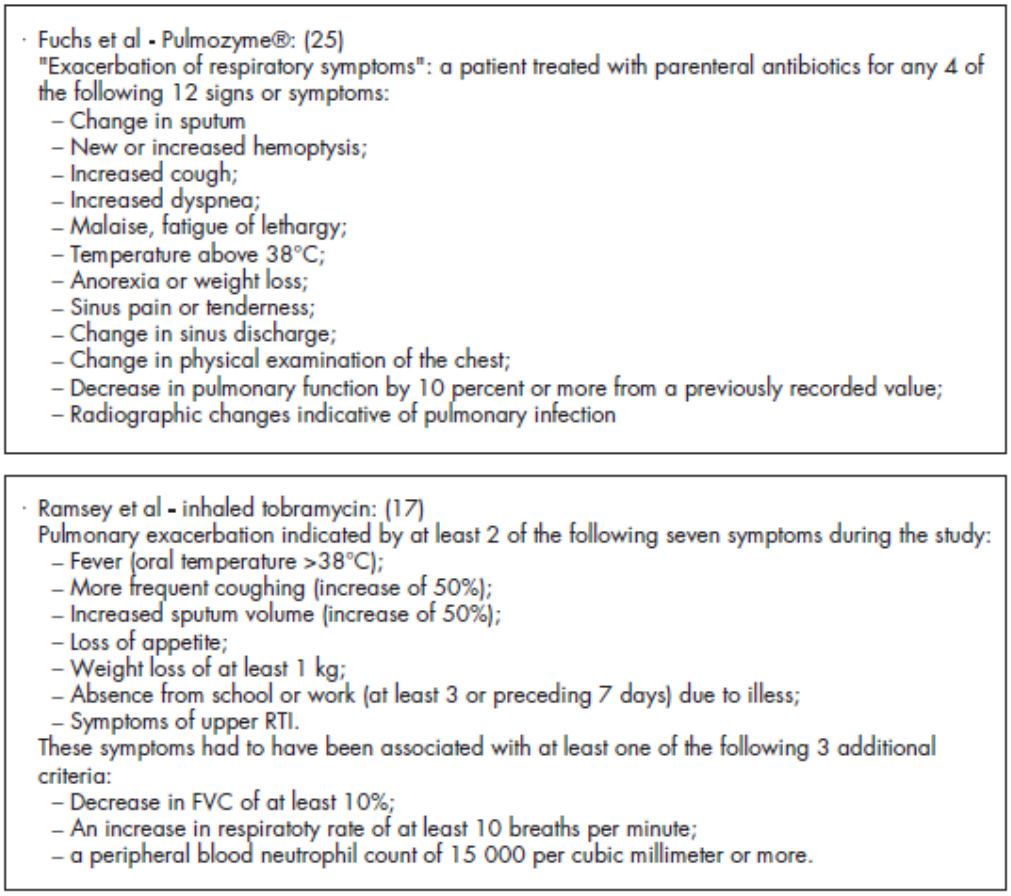

Figure 1-1 - Diagnostic Criteria for diagnosing CFPEs used by Fuchs *et al.* (1994) and Ramsey *et al.* (1999) (from Goss and Burns (2007), with permission).

Despite the central importance of CFPEs as an endpoint to trials and clinical outcomes, no set of diagnostic criteria have ever been prospectively validated. Rosenfeld *et al.* analysed components of the above definitions to see which clinical characteristics best predicted CFPEs (Rosenfeld *et al.* 2001). Using multivariate analysis of data from 246 patients enrolled in the placebo control arm of the 1999 inhaled tobramycin trial (1999) they set out to define a CFPE based on clinical parameters rather than decision to treat. Symptoms rather than physical examination or laboratory investigations were found to be more predictive of CFPEs. The associated odds ratios for the studied parameters are outlined Table 1-1 below.

Characteristic	Odds ratio	95% CI
Patient history*		
Increased cough	24.5	14.6, 41.3
Increased sputum production or chest congestion	24.5	14.3, 41.7
Decreased exercise tolerance or dyspnea with exertion	22.4	12.8, 39.2
Increased fatigue	15.2	8.1, 28.2
Decreased appetite	15.2	7.8, 29.8
Increased respiratory rate or dyspnea at rest	14.1	6.8, 29.0
Change in sputum appearance	11.4	6.1, 21.2
Fever	5.9	2.8, 12.7
School or work absenteeism	5.6	3.2, 9.7
Increased nasal congestion or drainage	4.6	2.9, 7.2
Physical findings and pulmonary function		
Retractions or use of accessory muscles	12.9	4.1, 40.5
Pharyngitis	5.8	2.6, 13.3
Cyanosis	4.7	1.6, 13.3
Wheeze, rales, or rhonchi on auscultation	4.3	2.8, 6.6
Rhinitis	2.4	1.5, 3.7
Weight loss ≥1 kg over past month	2.1	1.3, 3.2
Decline in FEV_1 >10% over past month	2.7	1.4, 5.1

From univariate logistic models that utilized data from visits at weeks 8 and 20 combined.
*By subject report, presence of characteristic within preceding 2 weeks.

Table 1-1. Odds ratios for association of clinical characteristics with the presence of a pulmonary exacerbation (from Rosenfeld *et al.* (2001), with permission).

Additional work has corroborated these findings. In the previously mentioned survey of Australian CF clinicians (Dakin *et al.* 2001) the three features that had the greatest consensus as either “useful” or “very useful” in diagnosing a CFPE were all symptoms rather than clinical signs (‘change in exercise capacity’, ‘change in activity level’, and ‘change in sputum production’).

One other study of note has studied the definition of CFPEs, this time using data from North America in the Epidemiologic Study of CF (Rabin *et al.* 2004). Retrospectively analysing available data from 11,692 CF patients (3,267 >18 years old), features most associated with the diagnosis of a CFPE in adults were decline in FEV1, new crackles, haemoptysis, and increased cough.

Quantifying Subjective Continuous Variables

One limitation in any attempt to define CF Pulmonary Exacerbations is that diagnosing, or defining, a CFPE requires the categorisation of a number of continuous variables in to discrete variables. Indeed the measurement of breathlessness is complex has been the subject of much study. A number of alternative tools to quantify dyspnoea have been used, including the Borg Scale (Borg 1982), the MRC dyspnoea score (Fletcher *et al.* 1959), and visual analogue scales (VAS) (Gift 1989a). Cullen and Rodak have undertaken a review of these measures, among others, and found the Borg Scale and VASs to be most widely applicable with good validity and reliability (Cullen & Rodak 2002). Others have demonstrated that there is a close link between VAS breathlessness scores and Borg scores (Morris *et al.* 2007).

Cough is another major feature of Cystic Fibrosis and related pulmonary exacerbations. It is a notoriously subjective symptom. In many cases external factors such as friends and family may influence the internal experience of a cough on quality of life. Coughing may not trouble the subject directly, but may disturb or upset those in close proximity for long periods. Thus this may be a factor that impacts on the subject indirectly. Other factors such as depression and anxiety may also influence, and be influenced by, cough experiences (Decalmer *et al.* 2007). Smith *et al.* found a stronger correlation between objective sound recording measures of cough and a cough VAS, than between objective measures and a six point 'Cough Score' (Smith *et al.* 2006b). The cough VAS also showed a significant response to treatment for CFPE, unlike the 6 point 'Cough Score'.

1.1.4 Aetiology

Despite our extensive and rapidly expanding knowledge of the basic cellular defect in the CF airway, little is know about the pathogenesis of pulmonary exacerbations (Goss & Burns 2007). The symptoms that occur with a CFPE are probably the result of complex interactions between host defences, airway microbial flora, psychological and environmental factors (Hodson & Geddes 2007). The airways of most adult CF patients are permanently infected with bacteria, most commonly P. aeruginosa (see Figure 1-3 below). At this point it is worth defining the terms 'colonised' and 'infected'. In general 'colonised' refers to the (persistent) presence of an organism without evidence that it has stimulated an inflammatory or immune response from the host; 'infected' refers to the situation when the presence of an organism is associated with an inflammatory or immune response (Denis *et al.* 2007).

A role for viruses?

It is not currently well understood what triggers the change in the delicate balance between host and endobronchial microbial flora. A number of factors have been suggested. Respiratory viruses have been implicated by a number of researchers. Efthimiou *et al.* reported that a group of CF adults who were deemed to be suffering a deterioration over the preceding one month were significantly more likely to have evidence of recent non-Pseudomonas infection than a group of adult CF patients deemed to be clinically stable (Efthimiou *et al.* 1984) These other organisms included *Mycoplasma pneumonia*, *Legionalla pneumophila*, *Coxiella burnetii* and a number of respiratory viruses. Such findings were based on serological methods of detection that have now been replaced by more sensitive and specific methods such as PCR (Jungkind 2001).

Wang *et al.* prospectively followed 49 teenage subjects with CF and 19 unaffected sibling controls. Although there were more reported respiratory symptoms in the CF group, there was an identical rate of detection of viruses in the two groups (Wang *et al.* 1984). Importantly however, in the CF group there was a highly statistically significant correlation between the annual incidence of upper respiratory tract viral infection and every measured marker of disease progression. However the viral detection methods used in the above studies were either tissue culture or immuno-fluorescence, both of which have since been shown to be relatively insensitive compared to the currently accepted gold standard of Nucleic Acid Amplification (NAA) (Punch *et al.* 2005). More recent studies employing such techniques have detected viruses in nearly half (46%) of exacerbations (Wat *et al.* 2008b), although it should be mentioned that this was a population with a mean age of 9, and the definition of 'exacerbation' was considerably different to other published definitions (see Figure 1-1 above). Despite the evidence for a role for respiratory viruses in CFPEs and clinical deteriorations, there is little evidence to show that vaccination or passive prophylaxis with monoclonal antibodies reduce exacerbation rates or improves outcomes (Giebels *et al.* 2008; Piedra *et al.* 2003; Wat *et al.* 2008a). It should be noted that these negative studies of vaccination were retrospective, and only contained small numbers of subjects. In addition there is considerable evidence that is suggestive of a significant role for viral infection in the development of chronic bacterial lower respiratory tract infections (Hament *et al.* 2004; Hament *et al.* 2005).

Viruses may play an indirect role in the development of chronic bacterial lower respiratory tract infection. In a mouse model of chronic pulmonary *P.*

aeruginosa infection, influenza infection enhanced susceptibility of the mouse to fatal Streptococcus pneumonia infection (Seki *et al.* 2004). In addition several studies have shown that there is no increase in the prevalence of viral respiratory tract infections in CF subjects compared to controls, but that such infections tend to be more severe and of longer duration (Hiatt *et al.* 1999; Ramsey *et al.* 1989; Van Ewijk *et al.* 2008; Wang *et al.* 1984). The reason for the increased severity of infections is not clear. It has been shown that there is no increased expression of IL-8 by *in vitro* bronchial epithelial cells that are deficient in CFTR (Black *et al.* 1998). However, there is evidence that respiratory viral infection may predispose to chronic *P. aeruginosa* infection, in effect 'opening the door' to allow *P. aeruginosa* to become established (Stark *et al.* 2006; Van Ewijk *et al.* 2006; Van Ewijk *et al.* 2007; Van Ewijk *et al.* 2008).

A role for new bacterial strains?

It has been demonstrated that the density of *P. aeruginosa* increases during a CFPE, and decreases during treatment (Regelmann *et al.* 1985; Regelmann *et al.* 1990; Smith *et al.* 1999). Furthermore, in Chronic Obstructive Pulmonary Disease (COPD), which is also characterised by chronic stable respiratory symptoms punctuated by 'infective' exacerbations, it has been shown that new strains of existing bacterial species are associated with pulmonary exacerbations (Sethi *et al.* 2002). Aaron and co-workers undertook prospective studies to determine whether new strains of bacteria were responsible for CFPEs in adults with CF (Aaron *et al.* 2004). They demonstrated that, in 34 of 36 adults chronically infected with *P. aeruginosa*, a single clonal species was found before, during, and after a CFPE, and that acquisition of a new strain was not responsible for the change in symptoms. They went on to demonstrate the

same principle in CF adults chronically infected with *Burkholderia cepacia* complex (*Bcc*) (Denis *et al.* 2007).

Recently, others have used a combination of culture-based and molecular techniques to assess microbial changes over time in subjects with CF. Sibley *et al.* performed T-RFLP in one subject over a period of time including three CFPEs (Sibley *et al.* 2008e). In addition to *P. aeruginosa* they identified a T-RF band consistent with bacteria from the *Streptococcus milleri* group (SMG). Following this they went to additional lengths to culture bacterial species from the same group in the same and additional CF subjects. They found bacteria from the SMG in 39% of samples taken at the time of CFPE, and in 7 of 18 CF subjects. They also reported clinical response to targeted treatment in subjects with high levels of SMG (by culture) who had failed to respond to more typical anti-pseudomonad treatment. Put together, the work of Sibley *et al.* adds to the body of evidence that microbial diversity beyond *P. aeruginosa* may be important in the aetiology of CFPE. The work has not yet been replicated beyond Canada, and has not included any clone sequence analysis.

Biofilm dispersal?

It has been hypothesised that a the biofilm is the natural state of growth for most if not all bacterial infections. Following from this it has been suggested that a CFPE may in fact be a manifestation of the "bacterial blooming" (Reid *et al.* 2006) of a mature biofilm, as part of a seeding process (VanDevanter & Van Dalfsen 2005). Support for this comes from clinical observations of a response to intravenous antibiotics (De Boeck & Breysem 1998; Moskowitz *et al.* 2004), but the *in vitro* biofilm resistance of *P. aeruginosa* isolates to the same antibiotics. In addition, mature biofilms have been shown to undergo such a

blooming process, known as seeding dispersal (Purevdorj-Gage, Costerton, & Stoodley 2005), *in vitro* flow cells (Kirov *et al.* 2007). However, others have cast doubt on the evidence to support such a hypothesis, with no correlation of *P. aeruginosa* CFU counts to clinical response or airways inflammation (Reid *et al.* 2006).

Non-microbial causes?

Other, non-microbiological factors have been associated with the incidence of CFPEs. Goss *et al.* conducted a cohort study of all CF sufferers aged greater than 9 years old registered in the American Cystic Fibrosis Foundation database (Goss *et al.* 2004). They demonstrated that living in a geographical area that had higher levels of particulate matter and ozone was associated with a significantly increased risk of exacerbation and lower lung function compared to those who lived in areas with cleaner air.

Schechter *et al.* investigated the role of socioeconomic class on outcomes for adult CF sufferers (Schechter & Margolis 1998). Using Medicaid status as a surrogate marker for socio-economic class in a retrospective analysis of the CFF database, they demonstrated that Medicaid patients were, among other findings, 1.6 times more likely to require treatment for a CFPE. Further work confirmed the initial findings (Schechter *et al.* 2009). This additional work suggested that factors such as health care utilisation only partially explained the discrepancies. In addition to this work from the USA, others made similar findings in UK CF populations, where an independent odds ratio of death of 2.75 in non-manual compared with manual social classes (Britton 1989). However, a smaller cohort study in relatively affluent Southwest England failed to demonstrate any such difference (Jarad *et al.* 2005).

Chronic pulmonary infection CF may theoretically result in a high degree of oxidative stress. A number of studies have highlighted a possible role for dietary deficiencies in the aetiology of CFPEs, possible mediated through deleterious effects oxidative / anti-oxidative balance. In a retrospective analysis of 597 CFPEs in 102 patients, confirmed in prospective analysis of a further 62 CFPEs, Hakim *et al.* demonstrated that, for patients with pancreatic insufficiency, having lower levels of either Vitamin A or Vitamin E were associated with an increased number of CFPEs. For CF patients who were pancreatic sufficient this was true for Vitamin A, but for Vitamin E the results did not quite reach statistical significance (Hakim *et al.* 2007). It should be noted that this effect held true, even for serum levels that are considered within the normal range. The mechanism for this is not clearly elucidated, but there is a body of evidence that highlights the role of Vitamin levels in adequate immunity to viral and bacterial pathogens (Beck 2007; Hakim *et al.* 2007; mit-Romach *et al.* 2008).

No study has yet demonstrated a significant survival benefit from vitamin supplementation. However, in a small pilot study RCT of 26 children (age range 7-18) Abdulhamid *et al.* demonstrated a reduction in the use of oral antibiotics in those whose diet was supplemented with zinc (Abdulhamid *et al.* 2008). This effect was only significant for those with low Zn levels at baseline.

In another RCT pilot study, Bruzzese *et al.* investigated a role for the probiotic *Lactobacillus rhamnosus* strain GG (LGG) (Bruzzese *et al.* 2007). There is evidence that the gastrointestinal tract of people with CF is constantly inflamed. LGG may have a role in a number of conditions, including chronic inflammatory bowel diseases (Doron, Snydman, & Gorbach 2005), and atopic diseases

(Kalliomaki *et al.* 2003). In the Bruzzese *et al.* study, a parallel cross-over design with 19 CF subjects in each group using oral rehydration salts or LGG supplements for six months before crossing over, significant improvements in body mass index (BMI) and FEV1 were demonstrated, and there were significant reductions in CFPEs and hospital exacerbations when taking LGG.

The link between nutritional status and survival in people with CF is well established (Elborn 2007). People with CF have a lower BMI than age matched control subjects. Using the American Cystic Fibrosis Foundation registry data, Liou *et al.* demonstrated that age adjusted weight *z* scores was negatively correlated with survival in a large well validated 5 year survival model (Liou *et al.* 2001). In order to maintain good lung health and reduce the incidence of CFPEs it is necessary to strive to attain a normal BMI.

Although it is uncontroversial to state that CF pulmonary disease is frequently polymicrobial (Vlamakis & Kolter 2005), there is a disproportionate body of work focused on understanding *P. aeruginosa* alone, compared to the body of work related to inter-species interactions. There are only limited publications that have attempted to address the mechanisms underlying species-species interactions directly relevant to CF (Duan *et al.* 2003; Hogan, Vik, & Kolter 2004; Mashburn *et al.* 2005; Sibley *et al.* 2008f). These studies have mainly focused on interactions between two species *in vivo*. Bearing in mind that previous work from, among others, Rogers *et al.* (2004) demonstrated a mean of at least 13 different species in adult CF sputum samples *in vivo*, there is potential for a level of interspecies complexity that has hitherto been entirely unsuspected and unstudied. There are a number of limitations to the species-species interactions studied *in vitro* above. Evidence for interactions comes from *in vitro* rather than

in vivo work. Thus there can be little certainty that the interactions described actually occur *in vivo* in the CF lung. A number of possible reasons exist that would limit the likelihood that the demonstrated interactions occur *in vivo*. Firstly *P. aeruginosa* is well known to exist primarily in biofilm form in chronic pulmonary infections in CF (Singh *et al.* 2000). Secondly it is thought that when *P. aeruginosa* grows in biofilms in the CF lung that it creates a micro-anaerobic environment (Worlitzsch *et al.* 2002). The interspecies interactions studied have been undertaken under aerobic conditions and work suggests that growing conditions can profoundly affect the phenotype of the bacteria (Hill *et al.* 2005). Finally, such simple two species interaction models fail to take in to account the additional complexity, both in communication and in competition for resources, that will be found when the multi-species nature of chronic CF pulmonary disease in taken in to account. It is well recognised that availability of bacterial micronutrients such as iron may have important implications on bacterial persistence and virulence (Harrison & Buckling 2009; Harrison *et al.* 2008; Hoffman *et al.* 2010) .

Despite the potential criticisms of these studies, they currently provide an insight into a world of potential inter-species interactions that has previously been unsuspected in CF microbiology. The interaction between *P. aeruginosa* and *Staphylococcus aureus* has long been suspected due to the inverse relationship seen in the colonisation rates of CF patients over time where *P. aeruginosa* rates increase with age while *Staphylococcus aureus* rates fall (see Figure 1-3 below). In addition, the work of Duan *et al.* has shown that the presence of the oft overlooked 'Oral Flora' in respiratory secretions can have significant implications for the virulence of *P. aeruginosa* in animal models

(Duan *et al.* 2003). There is as yet no work to assess the clinical relevance of these findings.

Other fields of microbial investigation beyond CF have progressed further. There is growing evidence for the role of bacterial intra- and inter-species signalling systems when, previously, unicellular bacteria were considered to be too primitive to be capable of such complex processes (Czaran & Hoekstra 2009; Prosser *et al.* 2007; Vlamakis & Kolter 2005). Such work may in turn inform the complex polymicrobial infections that are so characteristic of cystic fibrosis pulmonary disease.

1.1.5 Pulmonary Inflammation in Cystic Fibrosis

In addition to the overwhelming evidence for ineffective host immune response in CF, there are also laboratory and clinical data to suggest that mutant CTFR may be associated with a defective inflammatory modulation process (Buchanan *et al.* 2009), as outlined in Section 1.2.2 below. It is thought that cells deficient in effective CFTR have an exaggerated host inflammatory response, and that this in turn may lead on to an exaggerated inflammatory response *in vivo* to otherwise innocuous stimuli, microbial or otherwise. It has been hypothesised that the exaggerated inflammatory response may lead to excessive pulmonary destruction that is classical of CF. However, by the time most CF patients reach adulthood, chronic microbial pulmonary infection has occurred, with associated lung damage such as bronchiectasis, and resultant inflammation.

1.2 Airway defence and CFTR gene

1.2.1 Role of CFTR in airway defence against bacteria

The innate host defence to inhaled microbes is a multi-layered response that includes both chemical inactivation and mechanical clearance of invading pathogens. The chronic respiratory infections that lead to death in up to 90% of CF patients suggest that CF lung disease reflects a failure of defence against bacterial pathogens. One of the key mechanical mechanisms that aids host defence is the mucociliary apparatus (Wanner, Salathe, & O'Riordan 1996). The mucociliary apparatus has three main roles (Goldman *et al.* 1997; Travis *et al.* 1999). First it serves as a mechanical barrier that traps particles in the thin layer of surface liquid that covers the respiratory epithelium, then clearing them from the tracheo-bronchial tree by ciliary action (known as the mucociliary escalator), by a process of mucociliary clearance (MCC). Secondly the thin layer of liquid in which the cilia are bathed, known as airway surface liquid (ASL), acts as a chemical screen, neutralising pathogens through a number of secreted antimicrobial substances (Cole, Dewan, & Ganz 1999; Grubb, Jones, & Boucher 2004). Third mucociliary ASL acts as a biological and physical barrier to microorganisms, allowing interaction between host inflammatory cells and peptides, thus preventing adhesion or invasion of the respiratory epithelium (Matsui *et al.* 1998a).

Normal airway defences and mucociliary clearance are therefore dependent on ASL in a number of ways. ASL comprises two main compartments, the periciliary liquid layer (PCL), in which the cilia are bathed and allowed to beat freely, and the mucus layer which sits on top of the cilia and PCL (Garcia-Caballero *et al.* 2009; Wanner, Salathe, & O'Riordan 1996). The mucus layer is

moved caudally by the co-ordinated beating of the cilia. Only if the PCL is at the correct height and of the correct consistency to allow the cilia to beat, can mucociliary clearance occur.

Cystic Fibrosis was first postulated as a disease caused by mutation of a single gene by Lowe *et al.* (1949) based on the autosomal recessive pattern of disease inheritance. It took until 1989 before the gene was located and identified (Kerem *et al.* 1989; Riordan *et al.* 1989). The gene was named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. The CFTR gene is located on the long arm of chromosome 7 and covers an area of approximately 250,000 base pairs. The protein that the CFTR gene codes for consists of 1480 amino acids. This CFTR protein has been localised to the apical surface of epithelial cells (Rich *et al.* 1990) and spans the epithelial surface. Within the apical epithelial membrane, CFTR is found in close proximity to a number of other ion transport proteins, membrane receptors and the cytoskeleton (Short *et al.* 1998) (Ismailov *et al.* 1996). The role of the CFTR protein is primarily as a channel to transport chloride ion (Cl^-) from within airway epithelial in to ASL. Additionally it facilitates transport of sodium (Na^+) from ASL back in to the cell, via actions on the separate but co-located Epithelial Na^+ Channel (ENaC) (Myerburg *et al.* 2006). The presence of Cl^- and Na^+ in ASL osmotically draws water with it (Matsui *et al.* 1998b). CFTR tonically regulates ENaC Na^+ absorption in order to maintain a steady PCL height of 7 nm (Boucher 2004; Tarran *et al.* 2001). Thus a key role of CFTR is the maintenance of a normal ASL composition and height (Boucher 2007; Matsui *et al.* 1998b; Matsui *et al.* 2006; Tarran *et al.* 2001) In CF airways, failure of Na^+ absorption and failure of Cl^- secretion results in depletion in the volume of the

PCL (Matsui *et al.* 1998b). Depletion of the PCL leads to inadequate function of the cilia and failure of adequate mucociliary clearance (Matsui *et al.* 2005).

People with CF demonstrate a marked immunodeficiency to a number of airborne pathogens. CFTR may also play a role in innate cellular airway defence. A number of researchers, including Di *et al.*, have demonstrated that alveolar macrophages that are deficient in CFTR are unable to adequately acidify intracellular lysozymes (Di *et al.* 2006; Haggie & Verkman 2007). Consequently ingested bacteria are inadequately killed in CFTR deficient macrophages, allowing bacteria to persist, potentially contributing to additional infection and inflammation burden. This effect on phagocytic cells is uncertain. Moraes *et al.* demonstrated that, although the neutrophils are the most numerically abundant phagocytic cell of the innate immune system, abnormalities in CF ASL had no effect on neutrophil antimicrobial function (Moraes *et al.* 2006). More recently, others have challenged these conclusions showing that there may be functional and signalling changes in neutrophils in CF. In addition it has been shown that CFTR is expressed in large quantities on the surface of neutrophils (Painter *et al.* 2006).

1.2.2 Role of CFTR deficiency in inappropriate immune response

It is still unresolved whether infection precedes inflammation or *vice versa* in the new born CF airway (Armstrong *et al.* 1997; Armstrong *et al.* 1995; Armstrong *et al.* 2005). A number of *in vitro* studies have highlighted the possibility that a defect in CFTR itself may result in a dysregulated and overactive immune response without exogenous stimulation (Bonfield, Konstan, & Berger 1999; Hybiske *et al.* 2007; Tirouvanziam *et al.* 2000; Tirouvanziam, Khazaal, & Peault

2002). *Post mortem* studies on 8 CF neonates who died of non-respiratory illnesses showed macro- and microscopically normal lungs (Zuelzer & Newton 1949). However, these studies were undertaken 6 decades ago in limited numbers of patients without access to more advanced techniques. A more recent study of one set of lungs from a 24 week foetus with CF, which was terminated for unrelated reasons, showed macroscopically and microscopically normal lungs, but evidence of excessive inflammation as measured by a number of key inflammatory mediators (Verhaeghe *et al.* 2007). More recent studies in *ante mortem* subjects have attempted to determine whether there is more inflammation in very young children with CF but who have never had respiratory symptoms or requiring antibiotics. Armstrong *et al.* (1997; 1995) performed Broncho-Alveolar Lavage (BAL a sensitive technique for sampling the distal airways (British Thoracic Society. 2001; Mehta 1999) and alveolar spaces with less contamination from large airway mucus and micro-aspiration) on 45 infants with newly diagnosed CF. Importantly, 32 of the cases were identified by screening and 29 were free from any symptoms of respiratory infection. A group of 9 infants with no other illness or symptom other than stridor who were undergoing investigation by bronchoscopy provided control samples. When comparing the 'uninfected' CF group with the control group there was no difference in the mean level of Interleukin-8 (IL-8), a key inflammatory mediator. There was a very significant difference between the 'infected' group and both the control group and the 'uninfected' group. However other investigators have had different findings. Khan *et al.* showed that a variety of inflammatory markers, including IL-8 were raised in BAL samples from infants with CF who had culture negative samples (Khan *et al.* 1995). It should

be noted that no culture independent microbiological techniques were used in any of these studies.

In an attempt to circumvent the practical difficulties in interpreting the results of human *in vivo* work, a great deal of work has been performed on animal models and in cell culture models. Bonfield *et al.* in 1999 (Bonfield, Konstan, & Berger 1999) showed that mutant CFTR cell lines failed to produce any detectable levels of the anti-inflammatory interleukin IL-10, but excessive amounts of the pro-inflammatory cytokines IL-6 and IL-8, when compared to cell lines from healthy controls. Many others have also found *in vitro* evidence of excessive hyper-inflammatory innate immune response in CFTR deficient cell lines (Aldallal *et al.* 2002; Hybiske *et al.* 2007; Ribeiro *et al.* 2005) . However considerable controversy exists with regard to the validity of this data for two main reasons. The first is methodological and technical concerns regarding the nature of the *in vitro* work and the failure to replicate key findings (Aldallal *et al.* 2002). The second is the failure to convincingly demonstrate that inflammation precedes infection *in vivo* (Armstrong *et al.* 1997; Armstrong *et al.* 1995).

Much of the current understanding of healthy airway defence against microbial pathogens comes from research in to the defect caused by CF (Kreda *et al.* 2005).

There are essentially two competing theories to the link the genetic defect and the clinical picture. These can be divided in to the ‘high salt’ hypothesis and the ‘low volume’ hypothesis. The ‘high salt’ hypothesis states that abnormally high ionic concentrations in airway surface liquid affects the ability of ASL to inactivate inhaled matter, leading to failure to eradicate and eliminate inhaled microbes. Support for this hypothesis comes from findings that chemical

abnormalities in CF ASL result in inactivation of host antimicrobial peptides, known as defensins (Smith *et al.* 1996). This then allows bacteria to persist unchallenged in the ASL during the attempted mechanical clearance from the lung by the mucociliary escalator, leading to infection inflammation and lung damage (Cole, Dewan, & Ganz 1999; Goldman *et al.* 1997; Tesse *et al.* 2007).

The 'low volume' hypothesis argues that the mechanism by which functional CFTR protein deficiency leads to the clinical phenotype is via dehydration and loss of height in the periciliary layer of the ASL (Knowles & Boucher 2002; Mall *et al.* 2004; Matsui *et al.* 2005; Matsui *et al.* 1998b; Matsui *et al.* 2006).

In addition immune defence mechanisms have been implicated as defective in CF. Moskwa *et al.* demonstrated that respiratory epithelium generated hypothiocyanite in sufficient quantities to mediate bacterial killing. CF respiratory epithelium, however, was unable to generate sufficient quantities of hypothiocyanite, and thus was unable to adequately kill bacteria (Moskwa *et al.* 2007).

Recently, additional evidence has come to light, linking the CFTR mutation to increased pulmonary inflammation and hyper-susceptibility to *P. aeruginosa* infection, through ceramide levels. Teichgraber *et al.* showed that inadequate acidification of intracellular organelles results in build up of ceramide rafts and subsequent inflammation (Teichgraber *et al.* 2008). Most interestingly, there already exists an acid sphingomyelinase inhibitor with the potential to prevent this build up, in the form of amitriptyline (Becker *et al.* 2009).

1.2.3 CFTR as Bacterial Binding Site

Despite the abundant evidence of impaired local host defence, this does not offer an explanation as to why, among all the microbes, the CF airway is so prone to infection by *P. aeruginosa*. Indeed, so associated is the culture of *P. aeruginosa* with respiratory secretions in CF that its discovery is a key part of a published algorithm for the diagnosis of CF (De Boeck *et al.* 2006). A number of key discoveries have been made that may offer some explanation for this, and in turn offer better insights to pulmonary immune defence.

In 1997 Pier *et al.* (Pier *et al.* 1996) demonstrated that normal CFTR protein in the apical surface of human respiratory epithelium was involved in the clearance of *P. aeruginosa* from the lung by binding to and internalising the bacteria. Cells lacking functional CFTR failed to adequately clear *P. aeruginosa* and thus left the airway at risk of persistent *P. aeruginosa* infection. This provided evidence of a direct link between the primary genetic defect and the clinical disease phenotype. In addition to the role of CFTR as a potential receptor for *P. aeruginosa*, another potential receptor for pathogenic bacteria has been identified.

In addition to CFTR, other apical epithelial molecules may act as receptors for pathogenic bacteria. Imundo *et al.* (Imundo *et al.* 1995) showed that *P. aeruginosa* bound to CF epithelial cells. However this *P. aeruginosa* could be displaced by either free *P. aeruginosa* or free *Staphylococcus aureus* (*S. aureus*), both of which are well recognised airway pathogens in CF, but could not be displaced by *Escherichia coli* (*E. coli*; not recognised as a CF airway pathogen). Through a number of experiments they demonstrated that

asialoganglioside 1, a key glycosphingolipid component of eukaryotic cell walls, was responsible for the enhanced binding.

Further evidence for the relationship between *P. aeruginosa* and CFTR came in 2007 when MacEachran *et al.* (MacEachran *et al.* 2007) demonstrated that one strain of *P. aeruginosa* secretes a protein that inhibits the expression of CFTR on the apical surface of human respiratory epithelial cells. This protein, termed CFTR inhibitory factor (Cif), was shown to reduce apical membrane CFTR expression and lead to a concomitant reduction in CFTR Cl^- ion secretion. Such a mechanism would lead to a greater chance of persistence of *P. aeruginosa* in an airway already deficient in functioning CFTR levels. In addition there may be methods by which *P. aeruginosa* alters the internal epithelial structure to reduce the strength of epithelial tight junctions, thus allowing it persist (Kierbel *et al.* 2007).

1.2.4 Summary

It can be seen from the wealth of evidence that CF pulmonary disease is a manifestation of both chronic and intrinsic inflammation and chronic persistent infection through a considerable number of different but overlapping pathways. A role for bacteria in driving pulmonary damage is clear, but the relative importance of intrinsic inflammation compared to bacterially derived inflammation is not. In addition, most studies on the role of bacteria in inflammation have focused on a role of *P. aeruginosa* and an extremely limited range of other pathogens. There is, however, increasing evidence for much greater bacterial diversity in the CF airway (Bittar *et al.* 2008; Rogers *et al.* 2004), findings that has not been reflected in the *in vitro* studies reported above.

1.3 Microbiology Techniques

The ability to identify infectious agents involved in the pathogenesis of disease revolutionised human health (Newsom 2003). Since the days of Louis Pasteur and Robert Lister, the use of selective media plates to facilitate the growth of particular pre-determined bacteria or types of bacteria, whilst inhibiting or restricting the growth of others has been the mainstay of microbial identification and the investigation of infectious diseases. However, there are a number of potential drawbacks to these techniques. Firstly, by their very nature, they are selective. This means that there are some bacteria that are actively excluded from the possibility of identification. This may be a valid step in some circumstances, but only if the presence of the excluded bacteria has been shown to be of no clinical consequence. In order to demonstrate this, it is necessary to first identify these excluded bacteria before their exact role (or lack of it) can be determined. Thus a detailed understanding of the role of these excluded organisms cannot be achieved in this way.

It is therefore clear that culture for the detection and identification of bacteria has its limitations. There are a number of alternatives to culture based methods for detection and identification of bacterial species. The majority of these utilise the nucleic acid content of bacterial cells, and these techniques will now be discussed in more detail. There is an alternative to culture that does not rely on nucleic acid analysis. Gas-liquid chromatography utilises the volatile fatty acids released by certain bacteria, particularly anaerobes (Foweraker *et al.* 2005). Each species has a particular profile of volatile fatty acids which allows for a degree of differentiation between species. These techniques are not dependent on prior culture of the bacteria and thus avoid some of the limitations

noted above. However the technique has not gained widespread acceptance, perhaps due to its limited specificity, particularly in comparison to other alternatives to bacterial culture (Smith *et al.* 2003).

1.3.1 Culture-based techniques

Identification of bacteria has long relied on the classical technique of growing a bacterium under optimal conditions in isolation and in planktonic form. Physical and biochemical characteristics are observed, and typical patterns recognised that allow for identification. Such techniques are, however, prone to errors (McMenamin *et al.* 2000; van Belkum *et al.* 2000), and are no guarantee of exact and specific identification of species. Further more, the results of standard antibiotic sensitivity testing has also been shown to be unreliable in CF (Foweraker *et al.* 2005), and unhelpful in predicting clinical response to antimicrobial therapy (Smith *et al.* 2003).

Culture based techniques are still the only widely accepted and used method for routine bacterial detection and identification in clinical practise, but this is despite evidence for their lack utility, and because of the lack of a validated better alternative, rather than because of their proven worth. They form an integral part of the routine care of patients with CF. Guidelines from both the UK (Cystic Fibrosis Trust 2001) and USA (Yankaskas *et al.* 2004) recommend sputum culture every three months, for the detection of *P. aeruginosa, Burkholderia cepacia* complex, *S. aureus* and methycillin resistant *S. aureus* (MRSA). Because of the perceived importance of bacteria to CF lung disease, a greater number of selective media plates are routinely used, compared to other conditions associated with the presence of bacteria in the lower airways (Burns *et al.* 1998; Carson *et al.* 1988; Henry *et al.* 1997; Wong *et al.* 1984).

Guidelines include recommendations on sensitivity testing of *P. aeruginosa* to a panel of locally approved antibiotics. However, some researchers have cast doubt on the utility of routine sputum culture and sensitivity testing in the management of adults with CF. Foweraker *et al.* set out to test the reliability of routine culture and sensitivity testing of P. aeruginosa by clinical microbiology laboratories in the UK (Foweraker *et al.* 2005). Firstly they prepared four different suspensions of the same *P. aeruginosa* morphotype from 101 CF sputum samples taken during a pulmonary exacerbation. Susceptibility to 12 different agents was tested against each, using the disc diffusion method. Furthermore, the reproducibility of sensitivity testing was tested by preparing a standard sputum sample containing four morphotypes from one *P. aeruginosa* genotype, before culture and sensitivity testing by 8 scientists at one laboratory, and scientists at seven other accredited laboratories. Results showed a lack of correlation in culture and sensitivity results, either within a single *P. aeruginosa* colony, or between identical isolates tested a different laboratories.. Many of the antibiograms from different morphotypes of the same *P. aeruginosa* genotype were completely different from one another. In addition antibiograms, from a standardised sample, produced by different scientists in the same laboratory and by scientists in different laboratories gave markedly different results. The same group went on to assess combination testing against multiple resistant *P. aeruginosa* isolates, using Multiple Combination Bactericidal Testing (Foweraker *et al.* 2009). The results again showed poor internal reliability, and poor correlation with clinical outcome measures.

If *P. aeruginosa* is the organism responsible for CFPEs, and sputum culture is a reliable and useful test, then it should follow that results of *P. aeruginosa*

sensitivity testing should impact on clinical outcomes. Smith *et al.* (Smith *et al.* 2003) set out to retrospectively test this hypothesis by analysing results from the placebo arm of a large randomised controlled trial of nebulised tobramycin in CF (Ramsey *et al.* 1999). During the trial period all patients received the same two intravenous antibiotics for any CFPEs (ceftazidime and tobramycin). Fifty six subjects in the placebo arm had a pulmonary exacerbation. Change in lung function during treatment was used as a surrogate outcome marker for clinical improvement. All subjects had sputum samples cultured and *P. aeruginosa* was tested for susceptibility to the same antibiotics. If susceptibility testing was a useful test it would be expected that patients who harboured organisms resistant to the treatment antibiotics would not show a clinical response, while those who did respond would be those with susceptible organisms. The results however were at odds with the hypothesis. Whether the most prevalent *P. aeruginosa* strain or the most resistant *P. aeruginosa* was studied, subjects who showed a clinical deterioration (hypothesised to be those with resistant organisms) had sensitive organisms, whist those with resistant organisms (who would have been expected to show a clinical deterioration) showed adequate clinical response.

Such findings of a lack of clinical utility of antibiotic sensitivity testing are in keeping with the clinical experience of many adult CF physicians (personal communications). One theory to explain the lack of utility, despite an improvement during treatment for a CFPE is that standard susceptibility testing tests individual antibiotics *in vitro*, whereas *in vivo* antibiotics are given together and may therefore be acting synergistically. Aaron *et al.* set out to test the hypothesis that CFPEs would be better treated if antibiotics were chosen on the

basis of *in vitro* synergy testing against *P. aeruginosa* (Aaron *et al.* 2005). Two hundred and fifty one CF subjects, all infected with multiresistant gram negative bacteria, gave three monthly sputum samples for conventional culture and sensitivity testing and also for combination antibiotic susceptibility tests using Multiple Combination Bactericidal antibiotic Testing (MCBT). Subjects who developed a pulmonary exacerbation were randomised to receive a 14 day course of two antibiotics, chosen either on the basis of conventional sensitivity testing, or on the basis of MCBT. The primary outcome measure was time to next CFPE. After 132 CFPEs and 4.5 years, despite a logical hypothesis and a well executed RCT, no statistically significant difference in time to next CFPE was found between the two groups. The authors concluded that MCBT is no better than conventional sensitivity testing for the guiding antibiotic choice for CFPEs. One possible explanation for this may come from recent work by Foweraker *et al.* who showed that the reproducibility, reliability and clinical correlation of MCBT were poor (Foweraker *et al.* 2009).

A further major limitation of culture, beyond the consciously selective nature of the technique, is the long standing acceptance that the growth requirements of very many bacterial species cannot be adequately replicated in the laboratory. In 1959 the first English language report appeared confirming previous discrepancies between the detection of bacteria by culture and other techniques. Jannasch *et al.* (1959) demonstrated that far more bacteria were detectable by direct microscopy than could be detected by culture. In some samples as many as 9,700 times more bacteria were detected by direct microscopy than by culture plate methods. It should be noted that this report had been preceded by a report in Russian by AS Razumov in 1932 (Razumov

1932). Both reports studied samples from aquatic environments. However this extent of this discrepancy does not hold for all environments. It has been noted that the proportion of bacteria detectable by culture was as low as 0.1-1.0% in oligotrophic aquatic habitats [i.e. those with low nutrient content], but as high as 80-90% in eutrophic habitats [i.e. those with high nutrient content] (Staley & Konopka 1985). Although these early studies were from environmental microbiology, there were also early studies in clinical settings (Gibbons *et al.* 1963; Socransky *et al.* 1963) with similar findings. In spite of these findings, the methods of detecting and identifying bacteria by culture has remained the mainstay of clinical microbiology, despite the research and development of new culture independent molecular technologies to aid and augment culture based techniques (Nature Editorial 2006).

One possible mitigation for the 'great plate count anomaly' (Staley & Konopka 1985) as it has been termed, is that the loss of diversity detected by the different techniques was equally proportionate, *i.e.* that an equal amount of diversity was lost across the whole phylogenetic range, rather than some phylogenetic parts losing diversity disproportionately to other parts. Dunbar *et al.* (1999) showed that this was not the case, and that the degree of diversity loss during cultivation was different between different habitats.

1.3.2 Techniques for Molecular analysis of microbial diversity

It is, as yet, not possible to culture all bacteria species. In some environments such as soil it has been suggested that as many as 99 % of bacterial species observed under a microscope cannot be cultured by common laboratory techniques (Borneman & Triplett 1997; Pace 1997; Torsvik, Goksoyr, & Daae 1990). The microbiological significance of the uncultured 99 % has been

debated (Kirk *et al.* 2004). It has been suggested that the 1 % that can be cultured may represent the entire community and that the other 99 % are in a physiological state that does not allow for their culture, and thus of little of no significance. Others have shown that if more extensive attempts to culture the bacteria are made, more closely mimicking the natural environment, more but by no means all species are identified (Kaeberlein, Lewis, & Epstein 2002). It has been argued that no environment has ever been exhaustively sampled to the point that ever species present has been identified (Bell *et al.* 2005; Curtis *et al.* 2006; Curtis, Sloan, & Scannell 2002; Nusslein & Tiedje 1999; Prosser *et al.* 2007). In addition, other Kingdoms have been shown to display high levels of diversity (Singh *et al.* 2006).

There are a number of different methods for analysing bacterial diversity. Some of these will be outlined below.

1.3.2.1 *Limitations of molecular based techniques*

Molecular based techniques have been introduced to overcome some of the limitations of more traditional techniques. They are, however, not without limitations of their own. Firstly, most methods depend on the release of nucleic acid material from within bacterial cells by breaching the integrity of the cell wall in some way. However, for any given sample containing a variety of species, the efficiency of cell lysis will vary. If some cells, for example those with stronger cell walls, are incompletely lysed, compared to others with thinner walls that are more completely lysed, this will lead to different yields of nucleic acid product and inaccurate estimates of bacterial community composition (Wintzingerode F von, Gobel, & Stackebrandt 1997).

1.3.2.2 *Non-PCR molecular techniques*

1.3.2.2.1 Guanine plus Cytosine

Differences in the Guanine plus Cytosine (G+C) content of DNA can be used to study the differences between bacterial communities (Nusslein & Tiedje 1999). This is based on the knowledge that bacteria differ in their G+C content, and that taxonomically related groups of bacteria only differ by 3 % to 5 % (Tiedje *et al.* 1999). Although this method can only provide a coarse resolution of the bacterial community, it avoids a number of the pitfalls of PCR. Furthermore it includes all DNA present, is quantitative and can detect rare species not previously suspected (Clegg, Ritz, & Griffiths 2000). There do not appear to have been reports of G+C being used to assess bacterial diversity in clinically relevant situations.

1.3.2.2.2 Nucleic acid re-association and hybridisation

Deoxyribonucleic acid (DNA) re-association is a measure of genetic complexity of any given bacterial community, and has been used to estimate bacterial diversity (Torsvik, Goksoyr, & Daae 1990). Total DNA from a sample is extracted and then purified. This DNA is then denatured and then allowed to re-anneal. The rate at which it re-anneals is a measure of the genetic diversity of the sample: the more diverse the species in the sample the longer it will take of a one strand to be re-united with a complimentary strand. Although this technique avoids PCR-related biases, it offers little in the way of sensitivity and offers no information on species identification.

Nucleic acid hybridisation using specific probes is an important qualitative and quantitative technique for estimating bacterial diversity (Clegg, Ritz, & Griffiths 2000). Importantly these hybridisations can be done on DNA or RNA and can

also be done in situ on intact cells (Fluorescent *In Situ* Hybridisation - FISH) allowing three dimensional spatial distribution of organisms within samples to be ascertained. Formerly these hybridisations were done using oligonucleotide probes labelled with radioactive isotopes, but are now more commonly performed using fluorescent probes (Schramm *et al.* 1996). Another refinement is the use of Peptide Nucleic Acid (PNA-FISH) (Nielsen *et al.* 1991). PNA has a number of advantages over DNA probes for use in FISH. Firstly PNA is electrically uncharged, and thus the PNA/RNA hybrid is more stable than a DNA/RNA hybrid. Also PNA is more hydrophobic than DNA, thus favouring entry through a lipid-based cell wall. One drawback of FISH is that it is relatively insensitive. It requires bacteria to be present in relatively high quantities in order for the fluorescent probes to be detected. The probes can be designed to be species-specific or more general as is required, but specific probes can only be designed for species that have been previously detected and sequenced, thus limiting its utility in detection of new or unsuspected species (Kirk *et al.* 2004).

1.3.2.2.3 DNA microarrays

DNA microarrays consist of an arrayed series of microscopic dots of DNA oligonucleotides, called features, each containing pico-molar quantities of different specific DNA sequences. These features are arranged in a two dimensional grid on a glass or silicone slide, called a chip. Hybridisation of the DNA fragments to fluorescently labelled probes is detected by sophisticated and sensitive instruments and software. The make-up of the features can be many different targets including gene products of respiratory epithelium (Wright *et al.* 2006), specific bacterial genes and gene products (Hentzer *et al.* 2003b) and

bacteria. One limitation of the technique is that it is highly dependent on the quality of the chip, and, as the chips are costly, quality assurance and reproducibility are challenging to demonstrate.

DNA microarray can also be employed to assess bacterial diversity (Greene & Voordouw 2003). However, one clear limitation of this method for analysis of bacterial community composition is that the bacteria detected will be pre-determined by the features that are chosen for that particular microarray. Given the vast diversity of bacteria, no chip, no matter how large, can ever come close to detection of all possible bacteria. However, if the possible diversity is limited, or presumed to be limited, then a DNA microarray chip could be employed (Roth *et al.* 2004).

1.3.3 PCR techniques

The polymerase chain reaction (PCR) is an enzyme-driven, primer-mediated, temperature dependent process which allows for the replication of a specific, pre-determined sequence of DNA (Millar, Xu, & Moore 2007). PCR has been a major step forward in utilising and understanding the genetic information stored in nucleic acids of living creatures (White, Madej, & Persing 1992), allowing amplification and analysis of miniscule quantities of nucleic acid. Following acquisition, purification and amplification by PCR of nucleic acid from a given sample, a number of different techniques have been used to detect bacteria.

1.3.3.1 *Specific PCR*

Specific PCR is the simplest PCR technique, and is designed to detect specific target microbes (Coenye *et al.* 2005; McMenamin *et al.* 2000). It is the most widely PCR available technique in UK clinical diagnostic microbiology

laboratories (Millar, Xu, & Moore 2007). In order for specific PCR to be useful two there are two pre-requisites. Firstly, the genome of the bacterial species in question must have been sequenced. Secondly, the presence of the species in question must be suspected. It would not be practical for specific PCR to be deployed for all known species in order to analyse an entire bacterial community. Furthermore, new species, genera and even families are still being described, thus making this approach inherently selective as well as intrinsically impractical.

1.3.3.2 *The bacterial genome*

A number of PCR techniques use specific characteristics of the bacterial genome that warrant further discussion. All living cells contain mitochondria. Importantly, however, bacterial mitochondria differ from eukaryotic mitochondria. Bacterial mitochondria are composed of two subunits, termed the 30S subunit and the 50S subunit. Within the 30S subunit is a strand of ribosomal RNA (rRNA) that is termed the 16S rRNA strand. This 16S rRNA strand is specific to bacteria. Within the bacterial genome is a section of DNA that codes for this 16S rRNA. It is a sequence that is approximately 950 base pairs in length and is common to all bacteria. However, there are differences in its code between species allowing for differentiation of most species (Liu *et al.* 1997).

1.3.3.3 *Bacterial Clone Libraries*

After isolation of total community DNA from a given community of bacteria in a sample, this DNA is used as a template for PCR amplification of 16S rRNA genes with universal or genus specific primers (Liu *et al.* 1997). This is usually followed by construction of a clone library for genes encoding rRNA and the

rapid screening of the library based on sequence differences. Harris *et al.* have applied such techniques to clinical samples, and have demonstrated the ability to detect more bacteria in samples from sites of infection than standard culture techniques (Harris & Hartley 2003). However it was not clear what anatomical sites these samples came from. Van Belkum *et al.* have applied bacterial clone techniques to sputum samples from a limited number of CF patients (van Belkum *et al.* 2000). Using both general primers for gram negative and gram positive bacterial, as well as specific primers for species typically associated with CF pulmonary disease. They demonstrated a wide discrepancy between species identified by culture and those identified by PCR techniques. More recently Bittar *et al.* applied a more automated bacterial cone technique to a larger number of CF sputum samples (Bittar *et al.* 2008). They analysed 25 sputum samples from CF patients, and identified 13 different species by culture. They went on to generate and analyse 736 bacterial clones from the same samples. In this way they were able to identify 53 different species (mean 7.3 per specimen).

However, this technique remains a time and labour intensive process not well suited to relatively high throughput situations.

1.3.3.4 *Denaturing gradient gel electrophoresis*

Denaturing Gradient Gel Electrophoresis (DGGE) is a variant of another PCR-based technique, temperature gradient gel electrophoresis (TGGE). As with other techniques they require the prior purification and amplification of DNA. DGGE/TGGE utilise the physical properties of DNA. At room temperature, DNA is a stable negatively charged tightly wound double helix molecule. However, when the temperature is raised the strands start to become unwound from each

other. This process is termed denaturing. The degree to which the double helix is either partially or completely unwound depends on the temperature, and for any given sequence, the denaturing temperature will be different. Further more, because the molecule is negatively charged, it will move towards the positive electrode when placed in an electric field. In DGGE/TGGE, bacterial DNA is placed on an electrophoretic gel under appropriate denaturing conditions. The resultant pattern of bands is representative of the diversity of bacterial species in a given community (Pang *et al.* 2005; Tominaga 2006).

1.3.3.5 *Ribosomal intergenic spacer analysis (RISA)*

RISA provide ribosomal based fingerprinting of the bacterial community. RISA makes use of the intergenic spacer region between the 16S and 23S ribosomal subunit regions of the bacterial genome. This region is amplified by PCR, denatured and separated on a polyacrylamide gel under denaturing conditions. RISA has been applied to environmental (Fisher & Triplett 1999) and clinical situations (Scanlan *et al.* 2008), but not to respiratory samples. The technique is highly reproducible but time consuming. An automated variation (ARISA) offers faster turn around times and greater sensitivity (Fisher & Triplett 1999).

1.3.3.6 *Multi-Locus Sequence Typing*

Multi-locus sequence typing (MLST) is another PCR based technique which compares strains of the same bacterial species. It does this by amplifying and then sequencing sections of the bacterial genome encoding for well-preserved 'house-keeping' genes. Multiple house-keeping genes are amplified and sequenced to increase discriminatory power (Maiden *et al.* 1998). It was first used to discriminate different strains on *Neisseria meningitis*, but has since been applied to *P. aeruginosa* and *B. cepacia* strains in CF (Kidd *et al.* 2011;

Waine *et al.* 2007). It has revealed interesting adaptations of *P. aeruginosa* strains to the CF airways compared to environmental strains.

It is a powerful tool for strain typing within a species for epidemiological and infection control purposes. However, it is not well suited to analysis of complex multi-species bacterial community structure.

1.3.3.7 *Restriction fragment length polymorphism techniques*

Restriction fragment length polymorphism (RFLP) and terminal restriction length polymorphism (T-RFLP) are related PCR techniques. Common to both, PCR-amplified rDNA is digested by a restriction enzyme. In RFLP all the generated fragments are detected using agarose or non-denaturing polyacrylamide gel (Liu *et al.* 1997). RFLP is useful for screening clones or for detecting changes in bacterial communities. However, as any one species may generate as many as four to six restriction fragments, RFLP produces banding patterns that are too complex to be useful as a measure of diversity or for specific phylogenetic groups (Kirk *et al.* 2004). T-RFLP attempts to address the short comings of RFLP in bacterial community analysis. T-RFLP follows the same principle as RFLP except that the PCR primer is labelled with a fluorescent dye. When the products of PCR are digested using a restriction endonuclease, only the terminal fragments that are labelled with the fluorescent dye are detected (Liu *et al.* 1997). This considerably simplifies the banding patterns, making bacterial community analysis possible in even complex bacterial communities. In addition, each species only contributes one band to the result, so that the number of bands represents the minimum number of bands in the community. . See Figure 1-2 for an example of a T-RFLP gel, and post-T-RFLP processing to allow relative abundance calculations.

Each band on the polyacylamide gel represents a terminal restriction fragment of a certain length. It is possible for two or more species to share the same terminal fragment length, and therefore the presence of a band does not always identify a particular species. However, for T-RFLP the presence of a band can be said to represent *at least* one species, although the same is not true for RFLP.

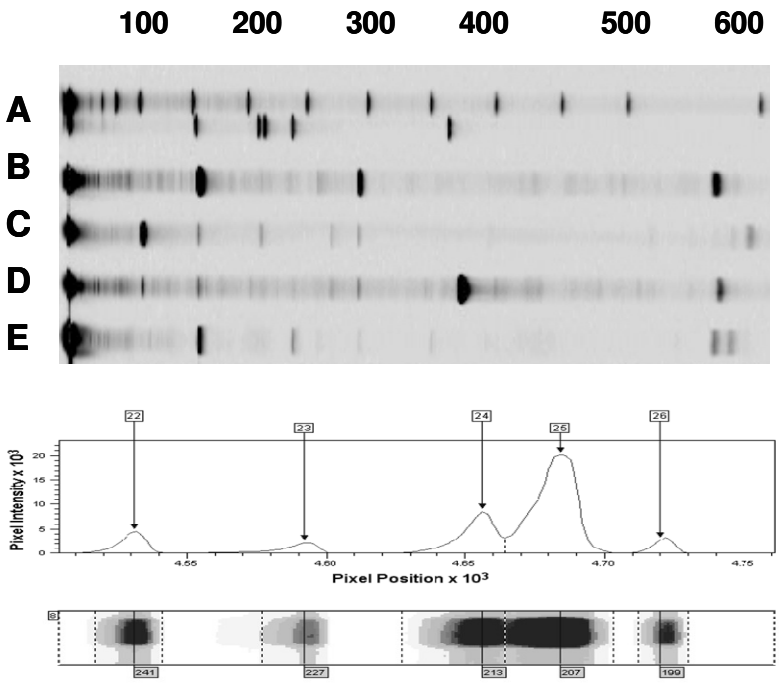

Figure 1-2 T-RFLP: example of polyacrylamide gel showing control ladder (top row - A), and four sample results (B-E). Lower images show conversion of band height, width and intensity into relative abundance. See text for more details.

In addition to T-RFLP, a technique performed on bacterial DNA, it is possible to add an additional step to the process. By performing a reverse transcription step prior to T-RFLP, it is possible to convert bacterial rRNA (manufactured by actively metabolising bacterial cells) to DNA. This added modification is called

RT-T-RFLP, and allows some assessment of the relative metabolic activity of the bacterial cells present (Rogers *et al.* 2005).

It has been demonstrated that the choice of restriction enzyme can influence results (Dunbar, Ticknor, & Kuske 2000). This is to be expected as different restriction enzymes will have different terminal cut points for any given 16S DNA fragment. For a complex community, more or less species may share a T-RF length, thus altering the apparent bacterial community structure. However, for temporal or spatial comparisons of a particular given bacterial community it remains a powerful tool (Dunbar, Ticknor, & Kuske 2000; Fourcans *et al.* 2007; Meier, Wehrli, & van, Jr. 2007; Pringault *et al.* 2007; Thies, Konig, & Konig 2007). Indeed Tiedje *et al.* reported five times greater success at detecting and tracking specific ribotypes using T-RFLP than using DGGE (Tiedje *et al.* 1999).

As a technique, T-RFLP has its caveats. Possibly the main potential bias in T-RFLP results concerns operon copy number heterogeneity (Klappenbach *et al.* 2001). Different bacterial species have different numbers of copies of the rRNA gene in their genome. The number of copies can range from one to 15. This could presents difficulties when interpreting the results based on quantification of the products of PCR that has targeted this gene in multiple different species (Crosby & Criddle 2003). However, as a technique, T-RFLP has been demonstrated to be valid for the analysis of temporal and spatial fluctuations in bacterial communities (Hartmann & Widmer 2008).

Since the inception of this study, a new advance and modification to the technique of T-RFLP has become available. Propidium monoazide (PMA) photo-cross linking was first described by Nocker *et al.* (2007). It utilizes the ability of PMA to bind to both extracellular nucleic acids and to permeate any

non-viable bacterial cell membranes to also bind to the nucleic acid present there. Following binding, PMA cross-links when exposed to light. The bound nucleic acid, cross-linked with PMA, is then easily removed from the solution. In this way, only nucleic acid from remaining viable cells remains. The PMA step within T-RFLP obviates the need for RT-T-RFLP to demonstrate the presence of viable and metabolically active. The technique has subsequently been successfully used on CF sputum in samples from this study (Rogers *et al.* 2008).

1.3.4 Taxonomic Ambiguity of Bacteria

One problem when considering the question of bacterial diversity in any environment is that there is no unified definition of what constitutes a species (Hey 2001). Traditional definitions of a species are based on higher organisms such as plants and animals and do not readily apply to prokaryotes (Godfray & Lawton 2001). Bacteria display a high degree of genetic plasticity that defies easy definitions of what constitutes a species. Whilst accepting that there is a degree of disagreement, most taxonomists would accept a percent identity score of $\geq$ 97% for classification to genus, and $\geq$ 99% for classification to species level, when using the 16S rRNA gene for identification (Petti 2007).

1.4 Microbiology of Pulmonary Disease in CF

There has been a longstanding belief that the range of pathogens found in CF lungs is limited (Govan & Nelson 1992; Lyczak, Cannon, & Pier 2002), and include bacteria such as *S. aureus*, *Haemophilus influenzae* (*H. influenzae*), *P. aeruginosa* and *Burkholderia cepacia* complex (*Bcc*). See Figure 1-3 below. Indeed, when only selective culture based techniques are used, species

detected are unsurprisingly, limited. Until recently such information has formed the basis of an understanding CF pulmonary microbiology.

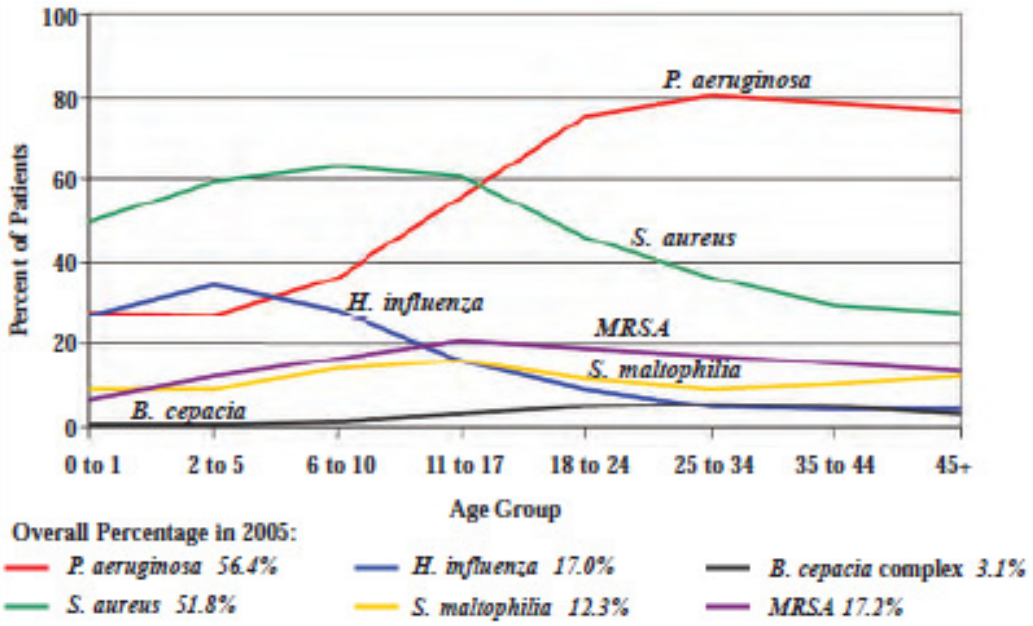

Figure 1-3 Respiratory Infections in CF. From 2005 CFF Patient Registry Report to Center Directors [with permission].

Historically these pathogens have been identified by culture-based techniques. From these culture-based surveillance data, a number of important messages can be determined. First of all, it can be seen that the rise of *P. aeruginosa* occurs after the peak incidence of *S. aureus*. Secondly, the rise in *P. aeruginosa* coincides with a fall in the incidence of isolation of *S. aureus*. Finally there is a degree of co-infection, with percentages adding up to greater than 100%. The traditionally accepted CF bacterial pathogens would thus be (in approximate order of acquisition): *S. aureus*, *H. influenzae*, *P. aeruginosa*, *Stenotrophomonas maltophilia* (*S. maltophilia*), *Bcc*.

This traditional view that the pathogens isolated from the CF sputum are limited in their diversity (Lyczak, Cannon, & Pier 2002) has been questioned through findings from both conventional and molecular biological methodologies (Bittar

et al. 2008; Rogers *et al.* 2003; Tunney *et al.* 2008). These methodologies have shown a marked range of phylogenetically diverse bacteria have been isolated. Although these discoveries have been of interest at the level of the individual CF patient, they have not as yet been shown to carry any wider morbidity or mortality implications. Furthermore, the techniques used have limited availability outside of the research laboratory.

Given the advances made in CF care based on the previously wide acceptance of a limited number of pathogens as important in CF pulmonary disease, a number of these will be discussed in more detail.

1.4.1 Recognised pathogens

1.4.1.1 *Staphylococcus aureus*

S. aureus was the first organism recognised as a pathogen of the CF airways. In the pre-antibiotic era few children survived beyond infancy and staphylococcal infection was the major cause of mortality (Anderson 1938). This lead to widespread use of anti-staphylococcal antibiotic prophylaxis. This in turn coincided with steady improvements in survival with CF. However, later retrospective studies suggested that such anti-staphylococcal treatments were associated with increased colonisation with *H. influenzae* and *P. aeruginosa*, and worse outcomes (Bauernfeind *et al.* 1987). Others have noted an association between *S. aureus* colonisation in adults and improved survival probability due to the beneficial effect of not being colonised with *P. aeruginosa* (Liou *et al.* 2001).

More recently controversy has surrounded the role of methicillin resistant *Staphylococcus aureus* (MRSA) in adults with CF. In both adult and paediatric

populations investigators have found a relationship between the isolation of MRSA from respiratory CF specimens and a worse clinical markers (Miall *et al.* 2001; Ren *et al.* 2007). However it has been suggested that such findings do not relate to increased pathogenicity of MRSA over and above MSSA, rather that those patients with MRSA are from a more unwell cohort, with consequently more exposure to healthcare institutions and thus at more at risk of contracting MRSA. More recently Dasenbrook *et al.* (Dasenbrook *et al.* 2008) attempted to control for such confounders by retrospectively analysing a very large cohort enrolled on to the (American) Cystic Fibrosis Foundation (CFF) patient registry. Subjects who became persistently MRSA positive in their sputum had a significantly faster rate in decline of FEV1, when controlling for other variables. This, however, does not prove causation, and it remains an unlikely possibility that MRSA is a marker for greater exposure to healthcare services.

1.4.1.2 *Haemophilus influenzae*

H. influenzae is commonly found as a commensal in the upper airways of healthy individuals (Moller *et al.* 1998). It is also frequently isolated from respiratory secretions in CF (see Figure 1-3 above), with a peak prevalence of around 35% in early childhood, declining to around 5% in later life (Gilligan 1991). However these reported figures may not be representative of the true prevalence for a number of reasons. Firstly culture of the relatively fastidious *H. influenzae* can be difficult in the presence of other more vigorous organisms (Govan & Nelson 1992). Despite early reports that *H. influenzae* does not persist between exacerbations of chronic respiratory tract illnesses (Govan & Nelson 1992), it is now recognised that the organism may be present in the

lower respiratory tract, evading host immune defences and antibiotics (Starner *et al.* 2006). In addition Moller *et al.* demonstrated that in explanted lungs from CF patients, *H. influenzae* was isolated in 10 of 16 CF patients, and was distributed throughout all divisions of the bronchial tree (Moller *et al.* 1998).

Additional factors that might contribute to an underestimation of the prevalence of *H. influenzae* by culture, as standard culture only employs aerobic conditions, and isolation of *H. influenzae* has been shown to be greatly improved by anaerobic culture (Gilligan 1991). Finally, *H. influenzae* has recently been shown to be able to exist and grow in biofilm phenotype in CF patients, a form which is inherently slower growing and more resistant to antimicrobial agents than the planktonic form (Starner *et al.* 2006; Starner *et al.* 2008).

The contribution of *H. influenzae* to the lung damage that is characteristic of CF lung disease has yet to be clearly delineated. When isolated in the context of a pulmonary exacerbation, it has been reported to be associated with higher levels of systemic inflammatory markers such as C-reactive protein (Govan & Nelson 1992).

1.4.1.3 *Pseudomonas aeruginosa*

P. aeruginosa is a gram negative, rod shaped bacterium that is a common environmental organism. In humans it is an opportunistic pathogen and in the respiratory tract it only causes disease if the host immune defence system is impaired in some way, for example in pre-existing chronic lung disease or in ventilator associated pneumonia (Sadikot *et al.* 2005).

Historically the improved survival of infants and children with CF that occurred with the advent of anti-staphylococcal therapy was followed by the emergence

of *P. aeruginosa* as the most important bacterial airway pathogen (Gilligan 1991). The prevalence of *P. aeruginosa* in cultured respiratory CF samples peaks at approximately 80% at around the age of 30 (see Figure 1-3 above). Understanding the interaction between *P. aeruginosa* and human airway epithelium defences has been a major focus for CF researchers for over four decades (Doggett, Harrison, & Wallis 1964; Hoffman *et al.* 2010). It is particularly adept at provoking an intense immune and evading host defences (Campodonico *et al.* 2008; Sabroe & Whyte 2007)

The success of *P. aeruginosa* is likely to at least in part due to its ability to form biofilms. Biofilms are defined as 'a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface' (Costerton, Stewart, & Greenberg 1999). Although the discovery of the biofilm mode of growth for bacteria did not occur until the 1970s some researchers have since hypothesised that the free swimming planktonic state exists primarily as a mechanism for migration of bacteria from one place to another and that biofilms are the *de facto* state (Watnick & Kolter 2000). Biofilms are a profoundly different mode of existence to the planktonic form. They are intrinsically slower growing, and are many orders of magnitude more resistant to antibiotics (Hill *et al.* 2005). For *P. aeruginosa* biofilms there is at least a six-fold up- or down-regulation of at least 800 genes when compared to planktonic growth, which is over 50% of the entire bacterial genome (Sauer *et al.* 2002). *P. aeruginosa* biofilms in CF were first identified by Singh *et al.* through the identification of quorum sensing (QS) molecules (Singh *et al.* 2000). Quorum Sensing is a mechanism of communication between individual bacteria that allows them to monitor the number of individuals in the vicinity, thus

allowing them to convert to biofilm growth at the optimum time (Bassler 2002). Other organisms have also been shown to be capable of forming biofilms, including in CF airways, such as *H. influenzae (Starner et al. 2006)*, S. aureus (Leid *et al.* 2002) and some fungal species (Hogan 2006; Loussert *et al.* 2009). Molecules that block the QS ability of bacteria have been suggested as possible future non-antibiotic therapeutic options for CF (Hentzer *et al.* 2003b; Hentzer *et al.* 2003a; Wu *et al.* 2004).

In a child or adult with CF the first positive culture of *Pseudomonas aeruginosa* is of considerable clinical significance. The age of onset of chronic *P. aeruginosa* infection is a predictor of survival (Frederiksen, Hoiby, & Koch 1998), and delaying the onset of chronic infection by aggressive antibiotic treatment prompted by detection of early isolates has been shown to improve lung function (Frederiksen, Hoiby, & Koch 1998; Littlewood *et al.* 1985). Antibiotic treatment with the aim of *P. aeruginosa* eradication with prolonged antibiotic therapy is thought to be successful in its aims (Taccetti *et al.* 2005; Valerius, Koch, & Hoiby 1991). It has been suggested that antibiotic strategies such as these can achieve eradication of *P. aeruginosa* from the lower respiratory tract in most subjects for a considerable period of time (Munck *et al.* 2001). However, in work by Taccetti *et al. (Boussaud et al. 2008)*, the definition of eradication was based on detection of *P. aeruginosa* by culture from throat swabs or sputum samples, and serum *P. aeruginosa* antibody titres. Both tests are considered insensitive marker of the presence of *P. aeruginosa* (da Silva Filho *et al.* 2007; Farrell & Govan 2006). Other data suggesting that eradication may indeed be possible comes from the analysis of the strains identified in respiratory samples at first isolation, and then at second isolation. Munck *et al.*

demonstrated that, in 14 of 19 cases, *P. aeruginosa* strains identified after an 'eradication' were different strains to those identified at first isolation (Munck *et al.* 2001). This still implies that, in 5/19 (26%) 'eradication' may not have been achieved.

Treatment for CFPEs is targeted at *P. aeruginosa* although it is not clear that it is the main cause of CFPEs (See CF Pulmonary Exacerbations on page 9 for more details).

1.4.1.4 *Burkholderia cepacia complex*

Burkholderia cepacia complex (*Bcc*) is a term that refers to a group of bacterial species. The *Bcc* is an important part of the rhizosphere as well as the wider environment. Its specific properties have been utilised in bio-remediation, and plant-growth promotion (Mahenthiralingam, Baldwin, & Dowson 2008). In particular, Bcc is also noted as an onion pathogen (Govan & Vandamme 1998). Initially the species in *Bcc* were considered as one species, in part due to the fact that biochemical testing was unable to tell them apart (Mahenthiralingam, Baldwin, & Vandamme 2002). The advent of molecular diagnostic allowed differentiation in to distinct species (also called genomovars). To date 17 genomovars (I – XVII) have been described (Cystic Fibrosis Trust 2010; Papaleo *et al.* 2010; Vanlaere *et al.* 2008; Vanlaere *et al.* 2009). The most clinically important is genomovar III (*B. cenocepacia*), which is the most frequently associated with a rapid clinical decline, as well as poor outcomes after lung transplantation (Boussaud *et al.* 2008; De Soyza *et al.* 2010; Hopkins *et al.* 2009). 'Cepacia syndrome', a devastating pulmonary infection in CF patients characterized by fevers, pulmonary infiltrates, rapid pulmonary decline and a high mortality rate, was first reported in 1984, from an outbreak in a CF

centre in Canada (Isles *et al.* 1984). Person-to-person transmission was demonstrated in 1990 (Lipuma *et al.* 1990), which led to the implementation of much more stringent infection control and segregation policies in CF units.

The mechanism by which *Bcc*, and particularly genomovar III exerts its marked detrimental effect, when other bacteria, typically more virulent in other clinical settings, fail to make such an impression is, as yet, not clear (Saldias & Valvano 2009). A large numbers of putative mechanisms have been investigated, that include genetic, transcription, secretory, protein, biofilm, and quorum sensing mechanisms.. Some of these include two transcription regulating proteins RpoE and RpoN, which are necessary to allow engulfed Bcc to delay phagosomal fusion and lysis (Flannagan & Valvano 2008), and for biofilm formation (Saldias *et al.* 2008). In addition, the biofilm quorum sensing system, *cepRI*, appears to be important to *Bcc* virulence, with *Bcc* CepRI knockout mutants showing reduced motility (Lewenza, Visser, & Sokol 2002), less stable biofilms (Tomlin *et al.* 2005), and reduced virulence in a rat lung model (Sokol *et al.* 2003).

The role of human anti-microbial peptides has previously been discussed in Section 1.2 above. *Bcc* possesses zinc metalloproteases which may give it the ability to cleave human anti-microbial peptides (Kooi & Sokol 2009).

Overall, *Bcc* is a virulent, transmissible, and difficult to treat infection with severe ramifications for CF patients.

1.4.2 CF as a Polymicrobial Pulmonary Disease

For people with CF lung disease it has long been recognised that more than one bacterial species may be identified, either from the same sample, or from

different samples from the same patient at different times. Even by the relatively insensitive and inaccurate methods of laboratory culture, it can be seen from the CFF registry data (See Figure 1-3 above) that the total vales for any one age group add up to more than 100%, indicating a significant degree of co-infection. As long ago as 1974 N. Hoiby reported co-infections in 'most patients' (Hoiby 1974). More recently Burns *et al.* reported finding a mean of 2.9 species per specimen by culture (Burns *et al.* 1998). Culture independent molecular methods are better suited to unbiased attempts to unravel the true diversity of bacteria in any environment (Kirk *et al.* 2004), not least in the CF lung (Bittar *et al.* 2008; Rogers *et al.* 2004). Using universal bacterial primers, Rogers *et al.* employed T-RFLP to analyse sputum samples from adults with CF (see 1.3.3 above. They found a mean of 13.3 (SD$\pm$7.9) species per sample (Rogers *et al.* 2004), and went to demonstrate, using RT-T-RFLP that most of species detected were metabolically active (Rogers *et al.* 2005). Although such techniques are not able to assign a species name with absolute certainty, Rogers *et al.*, and others, have used bacterial clone library analysis to confirm such diversity (Bittar *et al.* 2008; Harris *et al.* 2007). In work by Harris *et al.* the median age was 9.1 for the females and 8.5 for the males, and the lung disease severity was not reported. A large degree of diversity was, however, detected compared to both disease and healthy controls.

1.4.2.1 *The Role of Anaerobic Bacteria*

Recently, unequivocal evidence demonstrating that anaerobic bacteria are present in the lower airways of CF lungs has come to light. Tunney *et al.* used anaerobic culture of sputum and BAL fluid to demonstrated that 64% of samples from CF adults had high concentrations of species from the anaerobic genera

Prevotella, *Veillonella*, *Propionibacterium* and *Actinomyces* (Tunney *et al.* 2008). Anaerobes were also isolated in the majority of samples from healthy control volunteers, but at much lower concentrations and from different genera. The identification of *P. aeruginosa* from the same sample significantly increased the likelihood of also identifying anaerobes. This finding has since been challenged by Worlitzsch *et al.*, who, although finding obligate anaerobes by culture in 41 or 45 CF subjects, did not find an association with colonisation with *P. aeruginosa* (Worlitzsch *et al.* 2009).

As previously discussed, anaerobic culture is not routinely part of the panel of bacterial identification deployed in routine CF care (Cystic Fibrosis Trust 2002; Henry *et al.* 1997; Wong *et al.* 1984). The recent work by Tunney *et al.* is not the first evidence of anaerobes in the CF lung. In 1984 Thomassen *et al.* demonstrated that samples obtained from both the distal and proximal CF airway, obtained at thoracotomy, contained anaerobes in at least 20% of cases (Thomassen *et al.* 1984). Jewes *et al.* also demonstrated high concentrations of anaerobic bacteria in 24% of samples from 21 CF patients (Jewes & Spencer 1990). However, although efforts were made to distinguish between the presence of anaerobes in the lower respiratory tract and contamination by oropharyngeal anaerobes during passage through the oropharynx, there still remained concerns regarding the scientific validity of analysing expectorated samples for the presence of anaerobes; hence the importance of the anaerobes found in BALF by Tunney *et al.*, and by Harris *et al.*.

The work on anaerobic bacteria in CF sputum mentioned so far has relied on culture for detection and identification. Rogers *et al.* (Rogers *et al.* 2004)used T-RFLP to analyse CF sputum (see PCR techniques 1.3.3 ci-dessus). Although

T-RFLP does not allow identification with complete certainty, some small bacterial clone libraries were generated to strongly indicate that a significant proportion of anaerobes were present, including species such as *Bacteroides*, *Veillonella*, *Porphyromonas*, and *Prevotella*. It an attempt to demonstrate that such species were not due to oropharyngeal contamination mouth wash samples were also studied and compared to expectorated samples (Rogers *et al.* 2006). This added further weight to the evidence that the anaerobes were from the lower respiratory tract. More recently, Bittar *et al.* have undertaken much more extensive bacterial clone library analysis, generating 720 clones from 25 sputum samples, demonstrating 53 different species of which 16 (30%) were anaerobes (Bittar *et al.* 2008).

If there really are so many anaerobes in CF sputum where are the anaerobic environments in which they are finding an ecological foothold? Secondly, if *P. aeruginosa* is an aerobic species (Hoffman *et al.* 2006), but appears to be numerically dominant, how does it manage to inhabit a different ecological niche to the anaerobes? To help answer these questions a number of important pieces of work have been published, and in many areas, controversy remains. Firstly the biofilms which form the majority of bacterial growth in CF lower respiratory tract appear to be anaerobic environments. Worlitzch *et al.* demonstrated that, *in vitro*, a steep hypoxic gradient exists in thick CF sputum, in to which motile *P. aeruginosa* is able to penetrate before adapting to a biofilm mode of growth (Worlitzsch *et al.* 2002). Furthermore, Yoon *et al.* have demonstrated that *P. aeruginosa* is in fact capable of metabolising as a facultative anaerobe (Yoon *et al.* 2002). Although some ((Alvarez-Ortega & Harwood 2007)) have disputed these conclusions, concluding that *P.*

aeruginosa is in fact only metabolising in a micro-aerobic environment rather than an anaerobic one, the balance of evidence currently appears to favour *P. aeruginosa* as an anaerobic pathogen in the CF lung.

The clinical significance of the CF lung as a harbour for anaerobic bacteria is not yet clear. Tunney *et al.*, in their important work on anaerobes in CF sputum, tested antibiotic susceptibility patterns to the bacteria, both aerobic and anaerobic, that they detected (Tunney *et al.* 2008). Meropenem was the only antibiotic with Minimum Inhibitory Concentrations for 90% of isolates that fell within 'susceptible' for all species. All other antibiotics, including metronidazole which is typically considered to be effective against anaerobic infections (British Medical Association. & Royal Pharmaceutical Society of Great Britain. 2009), showed high levels of resistance.

1.4.2.2 *Significance of bacterial diversity*

What is the significance of this diversity? It is clear that a number of different bacterial species co-exist in close proximity to each other on the epithelial surface of the human lower respiratory tract in people with CF. However it also appears likely that such co-infection does not occur simultaneously, but changes over time, as both intra-pulmonary conditions shift, and as environmental exposure to potential community members continues. This principle of succession can be seen in Figure 1-3 on page 43 above. The potential for significant interactions between bacterial species, either directly through inter-species signalling, or indirectly through competition for limited resources, is great. Some work has been performed using *in vitro* culture of two or more species. For example *P. aeruginosa* has been implicated in the pathogenesis of *Bcc*, through up-regulation of *Bcc* virulence factors in the

presence of *P. aeruginosa.* Both species use N-acylhomoserine lactone (AHL) quorum sensing signalling molecules and have been shown to co-exist in mixed biofilms in the CF lung (Riedel *et al.* 2001). The study demonstrated that, *in vitro*, AHL from *P. aeruginosa* could be detected and influences *Bcc* but that AHL from *Bcc* had no effect on *P. aeruginosa*. Others have demonstrated similar communication between these two species, but additionally that the communication may in fact be bi-directional, if only for certain strains (Lewenza, Visser, & Sokol 2002).

When considering the importance of species diversity it is important to take account of the work done in different fields. Ecological study has been a scientific discipline long before such concepts were applied to bacteria. In order for any bacterial community to become established in a previously sterile environment, there must be some influx, or immigration, of bacteria. In the lungs, the normal host defence system is thought to prevent the establishment of a chronic bacterial community, and certainly prevents chronic inflammation and lung damage (See Section 1.1 above). For a chronic bacterial community to have become established some of the immigrant bacteria most become persistent. However, not all immigrant bacterial species will form part of the chronic bacterial community. For some species, conditions will not favour their persistence, and their presence in the environment in question will only be transient. Which species become established will be determined partly by deterministic factors such as the nature of the conditions, availability of substrate for the bacteria and the host response, and partly by stochastic factors (van der Gast, Ager, & Lilley 2008). Once a chronic bacterial community is established, the process of immigration will not stop. For new immigrant

species the presence of the established bacterial community will result in the environment being very different to that encountered by the first immigrants. Many of these later immigrants will be transient, but some may become established if they can find an ecological niche. When studying results of sampling of a community, it is valuable to be able to differentiate between transient species and resident species. Such analysis has been pioneered in other habitats by Magurran and Henderson (Magurran & Henderson 2003).

In ecological studies at a single time point it not possible to differentiate between species that are resident, *i.e.* permanent members of the community, from non-resident, *i.e.* transient species. These terms are ecologically synonymous with 'core' species and 'satellite' species. However, in data sets that are longitudinal it is possible to determine which species are core and which are satellite. Such an ability could have important implications in determining which species are the most relevant to the interaction between a community and its environment *i.e.* the human lungs, and are therefore more likely to play a role in chronic lung damage..

1.4.2.3 *P. aeruginosa and S. aureus Interactions*

There is a historical and temporal association between *S. aureus* and *P. aeruginosa* in the succession of CF pulmonary species (see section 1.4.1.1 above). Beyond this, Hoffman *et al.* demonstrated that there is communication between the two species *in vitro* (2006). Co-culture of the two species results in products of *P. aeruginosa* inhibiting respiration of *S. aureus.* Such inhibition leads to growth of a small colony variant (SCV) of *S.* aureus, which in turn has been shown to be intrinsically more resistant to key antibiotics used in clinical CF care such as tobramycin (Hoffman *et al.* 2006). Such findings could have

important clinical consequences, as antibiotics, particularly aminoglycoside antibiotics, are an extremely important treatment in CF pulmonary disease (Cystic Fibrosis Trust 2009a; Ramsey *et al.* 1999).

1.4.2.4 *P. aeruginosa and Fungal Interactions*

P. aeruginosa has been shown to have the potential to interact with organisms from different Kingdoms. It has been demonstrated that fungi from genera such as *Candida* and *Aspergillus* can be cultured from lower respiratory tract samples in patients with CF pulmonary disease (Burns *et al.* 1998). It is also common to find co-infection with *P. aeruginosa*. Both fungal species and bacterial species communicate with each other using small diffusible molecules, e.g. quorum sensing molecules. McAlester *et al.* demonstrated that communication between *Candida albicans* and *P. aeruginosa* is bi-directional (McAlester, O'Gara, & Morrissey 2008). A quorum sensing molecule (called 3-oxo-C12HSL) produced by some strains of *P. aeruginosa* affected the morphology of *Candida* albicans in a dose dependent fashion; and a molecule from *Candida albicans* (called Farnesol) limited the swarming ability of *P. aeruginosa*. Others have suggested that pyocyanin, a major inflammatory metabolite of *P. aeruginosa*, is able to inhibit growth of *Candida* and *Aspergillus* species (Kerr *et al.* 1999).

1.4.2.5 *Bacterial Competition for Nutrients*

Another area of potentially significant interaction between species is that of nutrient availability. In this area most work has focused on the role of iron as a nutrient for *P. aeruginosa*. Free extracellular iron in the lung can cause damage either directly, through oxidative free radicals, or indirectly by providing a vital food source for otherwise non-replicating bacteria (Moreau-Marquis *et al.* 2008).

Iron content of the CF airway is thought to be raised, possibly directly due to the defect in CFTR protein, and may provide an additional stimulus for *P. aeruginosa* to convert to its biofilm mode of growth, adding to aminoglycoside resistance. In addition, *S. aureus* may serve as a source of iron, providing *P. aeruginosa* with an additional stimulus to achieve chronic pulmonary infection in CF (Mashburn *et al.* 2005).

Since the discovery of iron as a key nutrient for *P. aeruginosa* growth, iron chelating has been explored for a potential therapeutic role in CF. Musk *et al.* explored the utility of 20 potential iron chelators for activity against *P. aeruginosa* biofilms *in vitro* (Musk, Jr. & Hergenrother 2008). After screening, potentially therapeutic compounds were tested for suitability to be delivered to the lungs by nebulisation. Two compounds (ferric picolinate and ferric acetohydroximate) passed both tests, and thus show potential for further *in vivo* investigational studies.

1.4.2.6 *In Vivo Studies of Bacterial Interactions*

Much of the work investigating the possibility of inter-species interactions in CF has been *in vitro* work. This *in vitro* work, although demonstrating that interspecies interactions are possible, does little to shed light on the tripartite relationship between different micro-organisms and host in the context of CF pulmonary disease. These short falls have been, in part, a necessity due to the difficulty of studying bacteria in situ within the CF lung. One group of investigators has attempted to circumvent some of these short comings by studying complex bacterial interactions in high throughput animal models. Although there are short falls in this approach, and results may not be directly applicable to humans, it does encompass some of the intrinsic complexity of

polymicrobial infections. Duan *et al.* used a rat agar bead model of CF pulmonary disease to investigate the interaction between oropharyngeal bacteria (oral flora – 'OF') and *P. aeruginosa* (Duan *et al.* 2003). Oral flora, in and of itself is part of the commensal microbial flora intrinsic to all humans. Oral flora include species from genera such as *Staphylococci*, *Streptococci*, *Veillonella*, *Porphyrmonas*, and *Prevotella* (Aas *et al.* 2005). Such species include both aerobes and obligate anaerobes. The study by Duan *et al.* study demonstrated marked *P. aeruginosa* virulence, when compared to OF when infecting the rat lung. However, when *P. aeruginosa* and OF co-infection occurred, *P. aeruginosa* numbers remained unchanged, but virulence increased very markedly. Molecular gene expression analysis demonstrated a number of key *P. aeruginosa* virulence genes that were up-regulated. Such works demonstrates the potential importance of bacterial that are frequently overlooked in clinical practice.

The same group has gone on to use an alternative animal, the *drosophila* (fruit fly) model, to allow higher throughput and faster results. Using this *drosophila* model, Sibley *et al.* investigated the nature of potential interactions between OF and *P. aeruginosa* (Sibley *et al.* 2008f). Using mortality of flies after a fixed time period as a surrogate marker of virulence, they were able to demonstrate that OF – *P. aeruginosa* interactions could be divided in to three broad groups, based on alterations in virulence: I) virulent alone, with increased virulence when co-infected; II) avirulent, even when co-infected with *P. aeruginosa*; and III) synergistic virulence, where killing of flies by OF occurred without *P. aeruginosa*, but was markedly enhanced by co-infection.

The bacterial interactions above specifically relate to species that may be some significance to CF. There has been work done that has investigated interactions between other species that may, or may not, be of significance to the polymicrobial situation in CF. Lysenko *et al.* used a mouse model to investigate the interaction, at the mucosal surface, between *H. influenzae* and *S. pneumoniae* (Lysenko *et al.* 2005). Both species were able to establish chronic colonisation at the mucosal surface when inoculated individually. However, when inoculated together, the result was chronic infection with *H. influenzae* and rapid clearance of the *S. pneumoniae*, associated with rapid influx of neutrophils. This finding provides an interesting insight in to the potential for species-species and host-species interactions, and how there may be unsuspected consequences when more than one species colonises a mammalian epithelial surface. This may have important implications for the direction of future therapeutic research in CF and beyond.

1.4.2.7 *Summary*

These studies demonstrate the potential for inter-species communication, but so far have only touched the surface of the potential complexity that may play a role in CF pulmonary disease. As previously discussed there may be 10 or more bacterial species in the lungs of one patient. There may also be a number of different ecological niches within any given lung, particularly in the more advanced stages of the lung disease. This may mean that the bacterial diversity estimates obtained so far are conservative. However it seems likely that many of these species will be living in close proximity, close enough to interact, and that these interactions may have clinical relevance.

1.5 Inflammation in Clinical CF Pulmonary Disease

The presence of bacteria such as *P. aeruginosa* are thought to stimulate the production of pro-inflammatory cytokines such as Interleukin-8 (IL-8) (Dean *et al.* 1993), and that endobronchial levels of such cytokines are raised in children and adults with CF. Furthermore, treatment for CFPE with antibiotics results in significant falls in endobronchial levels of the pro-inflammatory cytokine neutrophil elastase, as well as IL-8 (Ordonez *et al.* 2003). Others have demonstrated similar falls in sputum cytokine levels, correlating with falls in bacterial sputum density and serum markers of inflammation (Colombo *et al.* 2005). In addition, it has been demonstrated that sputum inflammatory mediators correlate to validated markers of disease severity such as FEV1 (Mayer-Hamblett *et al.* 2007).

Eosinophil Cationic Protein (ECP) has been shown to be present in high levels in both serum and sputum of patients with CF (Koller *et al.* 1996; Koller *et al.* 1997). Serum ECP levels correlated with sputum levels and in addition correlated to markers of pulmonary gas exchange (Virchow, Jr., Holscher, & Virchow, Sr. 1992). However, unlike levels of neutrophil elastase, levels of ECP were not reduced during treatment with antibiotics. In other chronic pulmonary diseases, such as COPD and asthma, ECP levels have been shown to be raised both in the chronic phase, and more so during exacerbations (Fujimoto *et al.* 1997; Gibson *et al.* 1998). In many situations, however, ECP appears to be related to allergy more than infection (Aldridge *et al.* 2002). Thus the role of ECP in CF pulmonary disease remains to be elucidated.

1.6 Summary

Despite major advances in our understanding of lung host defence, as well as many new treatments for CF, the great majority of people with CF continue to suffer with morbidity and mortality due to pulmonary infections. New insights in to the complexity of the symptoms and microbiology mean that efforts are required to further assess the *in vivo* mechanisms underlying CF pulmonary disease and exacerbations.

1.7 Hypothesis

For the purposes of this study it was hypothesised that, in the pathogenesis of CF pulmonary exacerbations, new bacterial species would be detected in, and otherwise disrupt, the stable bacterial community.

Chapter 2 - Materials and Methods

2.1 Clinical Trial

In order to assess changes in CF sputum before, during and after a CFPE, it was necessary to follow a cohort of volunteers, prospectively, over a period of time that included both CFPEs and periods of stability. In this way sputum samples and clinical data could be obtained, before either the symptoms of a CFPE began or treatment started.

The study was a cohort design that aimed to monitor longitudinal changes in symptoms, sputum bacteria and sputum inflammatory mediators. All volunteers received some or all of their CF care at the Department of Adult Cystic Fibrosis, Southampton University Hospitals Trust. The study was called 'Sputum Changes Over Time in Infective Exacerbations Of CF', or SCOTIE for short. Ethics committee approval for the study was obtained from the Southampton and South West Hants Local Research Ethics committee (06/Q1704/26). Written informed consent was obtained from all patients enrolled in the study. Dr. Mary Carroll, director of Southampton University Hospital NHS Trust adult CF service, supervised the study.

2.1.1 Study Duration

Each participant participated for 12 months. CFPEs are difficult to predict, but by attempting to recruit a cohort of volunteers who had exacerbated frequently in the previous 12 months, the chances of obtaining data from as many CFPEs as possible was maximised.

2.1.2 Sampling Frequency

It was necessary to achieve a balance between obtaining as many samples as possible so as to most accurately capture the dynamic changes during a CFPE, and the personal inconvenience to the participants, as well as the additional work and financial costs involved in such a strategy. It was decided that during periods of stability (in this instance defined as not taking additional antimicrobial therapy for a CFPE) samples would be collected on alternate days during the usual working week, i.e. on Mondays, Wednesdays and Fridays. Most participants provided samples during their normal morning routine of CF treatment on these days. However, some participants preferred to provide samples during the evenings with courier collection the following morning. These participants thus provided samples on Sundays, Tuesdays and Thursdays.

During CFPEs it was thought that the rate of any changes occurring in the CF lung would be greater. Thus, during a CFPE sampling frequency increased to daily. Daily sampling continued from the day antibiotic therapy started until the day it ceased. See Figure 2-1.

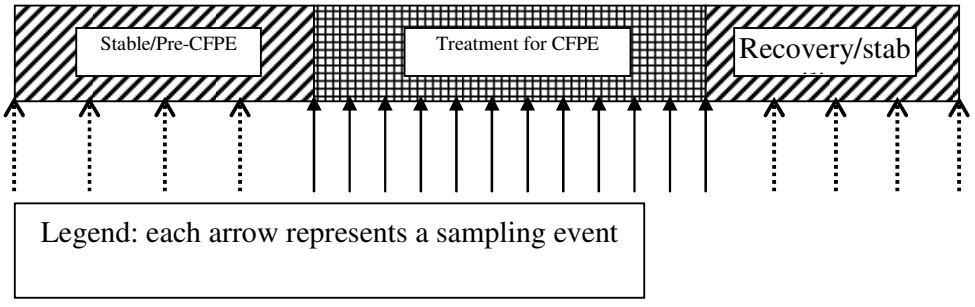

Figure 2-1 Sampling Frequency

2.1.3 Inclusion and Exclusion Criteria

The demands on participants' time due to the nature of the study design meant that a large number of the CF patients under the care of the Southampton Adult CF Unit would not be suitable. In order to obtain sputum samples from participants who were at home, it was necessary that each participant was able to spontaneously expectorate sputum most days. Furthermore, in order to study the mechanisms underlying CFPEs, participants would need to be likely to exacerbate during the course of the 12 months. CFPEs are difficult to predict. However recent work has been done to identify risk factors for CFPEs. Block et al (Block *et al.* 2006) studied CFPEs in 249 patients with multi-resistant organisms in their sputum. Fifty percent experienced a CFPE in the first year. Risk factors identified for developing a CFPE included younger age, female sex, lower FEV1, previous CFPE and the use of inhaled corticosteroids. Although the SCOTIE study was not primarily looking at multi-resistant organisms, the identification of risk factors allowed selection CF patients who would be most likely to exacerbate.

For the practicalities of transporting samples back to SGH it was also necessary that all participants lived within a distance of SGH that would allow the courier to return the samples within the allotted time. Furthermore, the transportation costs per sample increased considerably for collections further from the SGH hub. This meant that those CF patients living on the Isle of Wight or on the island of Jersey would not be suitable, as well as those living at greater distances from SGH on the mainland.

2.1.3.1 *Inclusion and Exclusion Criteria*

The following inclusion and exclusion criteria were selected. It was felt that these would provide a robust definition of CF, whilst making the practicalities of the study design realistic.

Inclusion

1. Aged 18 or over.
2. Confirmed diagnosis of Cystic Fibrosis.
3. CF genotype: either two copies of defective gene at position 508, or one copy at position 508 and one of the other 33 commonest recognised genetic defects.
4. Frequent exacerbators (ideally 3 or more exacerbations requiring antibiotic treatment in the last 12 months).
5. Clinically stable (no changes in antibiotic treatment in the last two weeks).
6. Geographically close to Southampton General Hospital (and excluding the Isle of Wight).
7. Some or all of their CF care received from the Adult CF Unit at Southampton General Hospital.

Exclusion Criteria

1. Currently having an Infective Exacerbation.
2. Previously received lung transplantation.

3. Unreliable hospital attendees (having missed 2 or more outpatient appointments without giving notice in the last 12 months).

2.1.4 Clinical Monitoring Form

It was necessary to prospectively collect data on the wellbeing of participants during the study. This fulfilled a number of objectives.

a) It allowed 'time to treatment' from the start of CFPE symptoms. This was also used to identify the time during which changes to the microbial community would be most likely to be found.

b) It allowed monitoring of 'time to recovery' during treatment for a CFPE.

c) It also allowed a grading of the severity for each CFPE.

d) It is part of the nature of PF pulmonary disease that symptoms and lung function fluctuate from day to day. Prospective monitoring of health status also allowed identification of fluctuations in health status that did not trigger treatment to be started for a CFPE. If these were shown to be of a similar magnitude to fluctuations that did trigger treatment, but recovered without treatment, then that may shed light on aetiology, and the role of treatments.

With each sputum sample that they provided, participants also provided data on their wellbeing. This data was captured by means of a specially designed sheet, termed Clinical Monitoring Form (CMF). See Appendix Section 9.1, page 274, for an example page. The CMF included four visual analogue scales, forced expiratory volume in the first second (FEV1), forced expiratory volume in

the six seconds (FEV6), oral temperature, a question about the presence of upper respiratory tract symptoms, and a section to track changes in antibiotic usage.

2.1.4.1 *Visual Analogue Scales*

A Visual Analogue Scale (VAS) is an instrument which attempts to measure a characteristic or symptom that constitutes a continuum of values and which cannot be easily measured (Grant *et al.* 1999). From a subject's perspective, this spectrum is continuous and cannot be easily categorised into discrete steps, as may be used in other scoring systems. As such, a VAS is a form of Patient Reported Outcome (PRO). PROs are increased recognised as an important tool in clinical CF research (Goss & Quittner 2007).

Each VAS used in this study constituted a horizontal black line 100mm in length, anchored at either end by a pair of descriptors representing opposite ends of the spectrum for that symptom. 'Best' was always at the left hand end and 'worst' at the right hand end for each VAS. The subject was required to indicate, by marking on the line, how they felt for that symptom at that time. The score was generated by measuring the distance, in millimetres, from the left hand end of the VAS to the mark made by the subject on the line. Thus a number was generated between '0' (='Best') and '100' ('Worst') for that symptom for that day (Gift 1989a; Kelsall *et al.* 2008; Wewers & Lowe 1990).

The four symptoms assessed on the Symptom Sheets for this study were:

a) Breathlessness

b) Cough

c) Sputum

d) General Wellbeing

These four symptoms were thought to best correlate with CFPEs, based on work by Rosenfeld *et al.*. (Rosenfeld *et al.* 2001).

2.1.4.2 *VAS Repeatability Testing*

In order to demonstrate whether subjects were reliably able to record their symptoms via the Visual Analogue Scales used as part of the CMF, measurement of the internal validity of the VAS was undertaken. Four subjects were asked to assist with this. Each of these four subjects was asked to complete the VAS parts of the CMF, before returning this to the investigator. Immediately after returning the CMF to the investigator, they were asked to repeat the exercise with a new CMF, before also returning this to the investigator. This exercise was repeated a total of four times, giving five completed CMF/VASs, for each of the four subjects.

2.1.4.3 *Spirometry*

FEV1 is the most widely used pulmonary function testing parameter of lung status used in CF (Gibson, Burns, & Ramsey 2003). This is for a number of reasons, including the wide availability of standardised spirometric equipment, accepted standards for performance, published validated reference values, inter- and intra-operator reproducibility and proven correlation to long term outcomes (Miller *et al.* 2005a; Miller *et al.* 2005b; Ramsey *et al.* 1999). FEV1 is also frequently used to attempt to objectively define the need for antibiotic therapy in clinical trials (Fuchs *et al.* 1994). Using serial measurements from the same person, a decline in lung function has been used as one of a number of indicators that the subject is experiencing a CFPE (Izbicki *et al.* 2000).

In this study, subjects measured their own spirometry each time they provided a sputum sample using a Koko Peak Pro [Ferraris Cardiorespiratory, Louisville, CO, USA]. This has been previously used in clinical studies in asthma (Wilson *et al.* 2006). Subjects were asked to record their FEV1 and FEV6 on their Clinical Monitoring Form. FEV6 was selected above FVC as FEV6 has been reported to be more acceptable to breathless subjects (Akpinar-Elci, Fedan, & Enright 2006) and has been shown to be equivalent to FVC (Jensen, Crapo, & Enright 2006; Jing *et al.* 2009). In this year long, longitudinal study, it was decided that a measure of spirometric obstruction that did not require a complete vital capacity manoeuvre would improve subject acceptability, adherence to the study protocol, and thus the quality of the data.

2.1.4.4 *Oral Temperature*

Acute infective episodes in any condition are commonly associated with a rise in body temperature. In some clinical trials in CF where CFPE have been an outcome measure, temperature >38°C has been one of the diagnostic criteria (Elkins *et al.* 2006). Furthermore, in one prospective study to identify risk factors for diagnosis of a CFPE fever was associated with an Odds Ratio of 5.9 (Rosenfeld *et al.* 2001).

In this study the oral temperature of each subject was assessed on each occasion that a sputum sample was provided. Temperature was measured using 3M™Tempa●DOT™ single use disposable thermometers [3M Healthcare, St. Paul, USA]. These thermometers were chosen for a number of reasons. Firstly they can be used sublingually in a way that is familiar to subjects (Macqueen 2001). They also allow for easy self-recording of temperature that could be awkward with other methods such as tympanic membrane thermometers. Thus no special technique is required. Secondly

they are single use and disposable, thus eliminating the chance of cross infection or misreading from previous attempts. Finally, the use of Tempa●DOT™ for measurement of oral temperature has been shown to be sensitive and specific compared to the historical standard of temperature using a mercury thermometer (Morley, Murray, & Whybrew 1998). The axillary route for measurement was rejected due to evidence of greater inaccuracy compared to oral measurements (Falzon *et al.* 2003). Compared to oral measurements, the axillary route has the added disadvantage of taking longer (3 minutes vs. 1 minute) (Macqueen 2001). The 'gold standard' rectal route was not considered for practical reasons. Infra-red aural thermometers were rejected due to concerns over the practicalities, and evidence of greater inaccuracy (Devrim *et al.* 2007).

2.1.4.5 *Changes to Antibiotic Therapy*

Any bacterial community is likely to be influenced in some way by antimicrobial therapy. CF patients with severe lung disease tend to be on a number of long term antibiotic therapies, such as inhaled colomycin, inhaled tobramycin, oral azithromycin or oral flucloxacillin. In order to study the effects of long term treatments and short term treatments on microbial communities and diversity it was necessary to be fully aware of all the antimicrobial therapy that each participant was taking at the time each sputum sample was provided. It was decide that the easiest way to do this was to take a complete medication history at the start of the study, and then ask participants to record any changes to their antibiotic treatment. This was achieved using the check box system seen on the CMF seen in Appendix 9.1.

2.1.5 Sputum Sample Collection Methods

As with most biological samples, it is generally accepted that the sooner a sample can be processed the more reliable and accurate the result. This is reflected in the Health Protection Agency Standard Operating Procedures (S.O.P.) (HSE Advisory Committee on Dangerous Pathogens 2005), which recommends that specimens should be transported and processed as soon as possible, but may be refrigerated for 2-3 hours "without appreciable loss of pathogens". However, given the longitudinal community-based sampling approach taken for the SCOTIE study it was not possible to meet these standards. The S.O.P. allows for culture of samples that are delayed by up to 48 hours, but advises that they should be refrigerated during the period of delay. Great care was taken with the design of the SCOTIE study to ensure that all samples were processed within 48 hours and were adequately refrigerated during the delay between production and processing. Adequate refrigeration was defined as a temperature between 2 ^{o}C and 6 ^{o}C inclusive.

2.1.5.1 *From Expectoration to Collection by Courier*

Subjects expectorated sputum as part of their normal daily routine of treatment and physiotherapy. They were provided with 60 ml sterile screw-top containers [Barlow Scientific, Stone, U.K.] for their samples. Sputum samples were then refrigerated at 2-6^{o}C. Domestic fridges were offered to all subjects for this purpose. The courier then arranged to collect each sample from subjects at a time that allowed the courier to ensure delivery of samples to the laboratory for processing within 36 hours. Although samples could be processed up to 48 hours after production according to the S.O.P. (HSE Advisory Committee on Dangerous Pathogens 2005), this 12 hour window allowed for handling time on receipt of samples in the laboratory, as well as providing a balance between the

practicalities of collecting and delivering large numbers of samples over a long period of time, and the aspirational scientific ideals.

It was not always possible for subjects to be present when the courier could collect their samples. Although this was generally discouraged, arrangements were made to allow for this. Subjects were provided with an insulated 'cool bag' and some freezer blocks [sourced locally]. Sputum samples could be placed with the pre-frozen [-18 ^{o}C] freezer blocks in the cool bag in a place accessible to the courier. This place had to be in a shady spot, and not in public view.

2.1.5.2 *From Collection to Delivery and Processing*

Transportation of biological samples by anyone other than the subject is closely regulated by the U.K. Health and Safety Executive report of 2005 (HSE Advisory Committee on Dangerous Pathogens 2005). All transportation of samples during the conduct of this research complied with those regulations. On collecting the sample from the subject, the courier transported the sample in an insulated specimen transportation bag [Versapak W-PYB1, Versapak, Erith, Kent, U.K.] that complied with UN3363 and PI650. Frozen cool blocks were provided to maintain the ambient temperature between 2-6^{o}C.

One further transportation stage was required. All samples were delivered to the HPA microbiology laboratory at SGH. Following culture, the remaining raw samples were frozen at –80oC prior to transportation at Kings College London for molecular analysis. Safeguards to the cold chain were used to ensure that frozen samples did not thaw during this stage of the transportation process. Samples were sent in large batches, in insulated transportation bags (conforming to PI650), and dry ice pellets were used to completely surround the

samples. A professional courier was used for all transportation of samples (OTR Couriers, 18 Hunters Crescent, Totton, Southampton, SO40 7FA).

2.1.5.3 *Auditing of Specimen Delivery*

In order to ensure that these standards were adhered to, a number of methods were employed to ensure standards.

2.1.5.3.1 Time to Laboratory

Each sputum sample was accompanied with a hand written log of the time and date of sample production (completed by the subject), the time and date of collection by the courier, and the time and date of delivery to the laboratory (both completed by the courier). If necessary, the time of delivery to the laboratory could be corroborated by cross checking with the laboratory computer records.

2.1.5.3.2 Temperature Chain

In order to monitor the ambient temperature of samples during the collection and delivery process, a small temperature recording device was used [ThermoData™ Logger; Electronic Temperature Instruments, Worthing, West Sussex, U.K.]. This recorded, but did not display, temperature recordings every 2 minutes from activation by the user for up to 72 hours. Data was subsequently uploaded to a personal computer for analysis using the ThermoData™ Logger software v 2.0.3.

2.1.5.4 *Effects of refrigeration time on measures of microbial diversity*

An integral part of the protocol for longitudinal sputum collection (see section 2.1.5 above) was collection and transportation of sputum samples from subjects

in the community to the base hospital. This was done in a timely fashion, and samples were kept chilled at all times in keeping with established protocols. Inherent to this process were short delays between expectoration and initial processing. The protocol outlined above allowed for delays of up to 36 hours between sample production to sample delivery and 48 hours from sample production and sample processing. However, prior to the undertaking of this study, the effects of short delays such as these on bacteria in the samples was not known. In order to ascertain the effects on the ability to detect bacteria, as well as their relative abundances, a small additional study was undertaken.

Freshly expectorated samples were collected and immediately separated from any adherent saliva. Sputum was then washed three times with 0.9% PBS before being placed on a sterile empty Petri dish. The sample was then physically homogenised using a sterile scalpel and forceps, before being separated into 6 separate aliquots. One aliquot was immediately frozen at -80^{o}C (called sample T0). Three samples were stored at +4oC for 18 hours prior to freezing at -80^{o}C; one sample was stored at +4^{o}C for 36 hours prior to freezing at -80^{o}C; and finally one sample was stored +4^{o}C for 72 hours prior to freezing at -80^{o}C. Four degrees centigrade was chosen as representative of the usual refrigeration temperature. The times of 18 hours, 36 hours and 72 hours were chosen as representative of, respectively, the median time to delivery of samples, the maximum acceptable time of delivery of samples, and an outlying value, most likely to show variation from T zero.

2.2 Sputum Microbiology

The methods used for collection and delivery of samples to the Health Protection Agency laboratory at Southampton General Hospital are detailed in Sputum Sample Collection Methods 2.1.5 above.

2.2.1 Laboratory Culture Techniques

All sputum samples were cultured in the clinical microbiology laboratory under the auspices of the Health Protection Agency at Southampton General Hospital. HPA standard operating procedures (Health Protection Agency 2004) for handling and processing clinical respiratory samples were followed throughout. This work was done by Biomedical Scientists working within the framework of the research team. Thus samples were processed in an identical manner to those samples received from CF patients as part of their standard clinical care.

2.2.1.1 *Sputum Culture*

Samples were first assessed for suitability for processing based on appearance. Samples that appeared entirely composed of saliva without mucopurulent sputum were rejected as inadequate. Samples were then physically homogenised using a sterile loop, before a small representative aliquot was placed on each of seven different culture plates. All culture plates were obtained from E & O Laboratories [E & O Laboratories Ltd., Burnhouse, Bonnybridge, Scotland. FK4 2HH].

The standard culture plates, specific incubation conditions and intended selected bacteria are detailed in Table 2-1 Media used for the culture of sputum samples below.

Microbial species identification was then undertaken, according to the chemical and physical properties of the isolates (Brenner *et al.* 2005; Health Protection Agency 2004).

In addition to microbial identification, the abundance of the isolate on the culture plate was determined in a semi-quantitative manner, in to three categories: ‘Scanty growth (+)’; ‘Moderate growth (++)’; and ‘Heavy growth (+++)’.

Name	Incubation Conditions	Example bacteria cultured
1. Blood Agar	5% Carbon Dioxide; 35°C; Optochin disc	Streptococcus pneumoniae
2. Chocolate Agar	5% Carbon Dioxide; 35°C; bacitracin disc	Haemophilus influenzae
3. Cepacia Plate	Aerobically; 35 °C	Burkholderia cepacia
4. CNA	Aerobically; 35 °C	*Staphlococci* and *streptococci*
5. PYO	Aerobically; 35 °C	*Pseudomonas* species
6. Sabouraud agar	Aerobically; 35 °C	Fungi
7. Cysteine Lactose Deficient (CLED)	Aerobically; 35 °C	Enterobacteriaceae

Table 2-1 Media used for the culture of sputum samples (COPD samples 1-2; CF samples 1-7)

2.3 Molecular Microbial Analysis

Sputum samples were collected, processed and transferred to Kings College London as described in Sputum Sample Collection Methods 2.1.5 above. All

T-RFLP work was carried out at Kings College London (see Acknowledgements on page x)

It should be noted that there were scientific advances that occurred during the course of the study that were incorporated in to the methods to improve the performance of the techniques. One such technique was the use of Propidium Monoazide (PMA) extracellular nucleic acid photo-crosslinking, the principles of which are discussed in more detail in Section 1.

2.3.1 Nucleic Acid Extraction

Prior to nucleic acid extraction, sputum samples were washed in a 5 × volume of phosphate buffered saline (PBS) to remove saliva. The sputum bolus was disrupted by the addition of 0.2% w/v solution of dithiothreitol (DTT) with incubation at room temperature for 15 min. Bacterial cells were pelleted by centrifugation at 5000 × g for 5 min. The pellet was re-suspended in 10 ml of 200 mM (pH 8.0) PBS and re-pelleted by centrifugation at 5000 × g for 5 min. This was repeated 3 times. Equal volumes (500 µl) of sputum or bacterial suspensions, PBS and Guanidinium thiocyanate-EDTA-Sarkosyl (GES) were added to 1.5 ml screw-cap tubes (Sarstedt, Leicester, UK) that contained one 3 mm tungsten carbide bead and 0.2 g of 0.2 mm acid-washed glass beads (Sigma-Aldrich, Gillingham, UK). Samples were homogenised for 60 s at 30 Hz in a Mixer Mill 300 (Qiagen, Crawley, United Kingdom).

Cell debris was pelleted by centrifugation at 12,000 x *g* for 3 min at 4^{o}C. Supernatant was transferred to a fresh non-stick RNase-free microfuge tube (Ambion, Applied Biosystems, Warrington, UK) that contained pre-aliquoted and cooled to 4 ^{o}C polyethylene glycol (to a final concentration of 15%) and NaCl (to a final concentration of 0.5 mM). After mixing, nucleic acids were left to

precipitate at -20°C for 5 min, followed by precipitation at 4 °C in a chilled centrifuge (Wolflabs, York, UK) at 12,000 x *g* for 20 min. Supernatants were removed and the nucleic acid pellets re-suspended in 500µl nuclease-free water (Ambion), vortexing for 2 s. To remove protein contaminants an equal volume of saturated phenol (pH6.6) (Fisher Scientific, Loughborough) was added, samples were vortexed for 10 s and left at room temperature for 1 min. Phases were separated by centrifugation at 12,000 x *g* for 4 min at 4°C. The upper aqueous phase was transferred to a fresh micro-centrifuge tube and 500 µl phenol: chloroform: isoamylalcohol (125:24:1, pH 4.3) (Fisher Scientific) was added. Samples were vortexed vigorously and phases separated by centrifugation at 12,000 x *g* for 4 min at 4°C. The supernatants were separated into two aliquots for separate RNA (400µl) and DNA (100 µl) precipitation.

Prior to DNA precipitation, some samples underwent treatment with PMA, as detailed below. Meanwhile other samples, analysed earlier in a separate part of the study, did not and went directly on to nucleic acid precipitation. See Section 1.3.3.7 above for more details.

Propidium monoazide cross-linking

DNA cross-linking using PMA was performed as described previously by Rogers *et al.* (Rogers *et al.* 2008). PMA was dissolved in 20% dimethyl sulfoxide to create a stock concentration of 20 mM with this added to 100 µl of sample to give a final concentration of 50 µM. Following an incubation period of 30 min in the dark with occasional mixing, samples were transferred to 24 well, flat-bottomed, cell culture cluster plates (Corning, New York, USA) for exposure to light. Samples were exposed for 3 min using a 500W halogen light source (AC220-240V/50Hz halogen floodlight; RS, Northampton, UK) at a distance of

20 cm. During this process, cell culture plates were placed on top of ice, with occasional shaking to guarantee homogeneous light exposure. After photo-induced cross-linking, cells were transferred to 1.5ml microfuge tubes and pelleted at 5,000 x *g* for 5 min prior to DNA isolation.

DNA precipitation

DNA was precipitated at -20^{o}C for 30 min after addition of an equal volume isopropanol (Sigma-Aldrich), 0.1 volume of 10 M ammonium acetate and 1 µl linear polyacrylamide (GenElute™-LPA, Sigma-Aldrich, Gillingham, UK). DNA was pelleted at 12,000 x *g* for 5 min and washed twice in 1 ml of 70% ethanol, dried at 37^{o}C for 5 min and re-suspended in 50 µl nuclease-free water.

RNA precipitation

Total RNA was precipitated in an equal volume of lithium chloride (final concentration 4M) (Sigma) for 30 min at -20^{o}C, pelleted at 12,000 x *g* for 2 min at 4^{o}C, washed once in 1 ml 70% ethanol (ice-cold), dried and re-suspended in 10 µl nuclease-free water (Ambion) by gently mixing with a pipette. Any contaminating DNA was removed with the Ambion Turbo-DNA-free™ kit according to manufacturer's instructions for rigorous DNase treatment (37^{o}C for 30 min with the addition of 1 U of Turbo-DNase at the beginning and after 30 min).

Reverse Transcription

High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) was used for the generation of cDNA according to manufacturer's instructions. In a 20 µl reaction, 2 µl of 10 x Reverse Transcriptase buffer, 0.8 µl of deoxynucleotide triphosphates (dNTPs) (to a final concentration of 5 mM),

primer 926r to a final concentration of 5 µM, 1 µl Multiscribe Reverse Transcriptase, 1 µl RNase Inhibitor and 300 ng of RNA were mixed. RNA was reverse transcribed in a GeneAmp PCR System 2400 (Perkin-Elmer, Beaconsfield, UK) at 25 ^{o}C for 10 min, 37 ^{o}C for 120 min, and 85 ^{o}C for 5 s.

2.3.2 *In silico* sequence analysis

In order to establish candidate species that were detected as bands in the sputum samples using the methods outlined above, published bacterial 16S rRNA gene sequence data, stored online at Genbank (http://www.ncbi.nlm.nih.gov/sites/entrez?keywords=db_Nucleotide), were retrieved. Mapsort (Wisconsin package version 10.3; Accelrys) was used to predict the band sizes for T-RFLP analysis. Mapsort, which locates the position of restriction endonuclease recognition motifs in a given sequence, was used to determine the length (in bases) from the 5' end of primer 8f-700IR (see below) to the first cleavage position of the endonuclease *Cfo*I in each of the Bacterial 16S rRNA gene sequences retrieved from Genbank. This process was performed on all the bacterial entries in the Genbank database that spanned the amplified region. In this way, it was possible to predict the length of T-RF bands generated from 853 separate phylotypes.

2.3.2.1 *T-RF Band Length Note*

In T-RFLP results of a number of subjects it was noted that two T-RFs dominated. These were T-RF 155 and T-RF 567. T-RF 155 is well characterised as the expected T-RF for *P. aeruginosa*. T-RF 567 is a band that is not associated with a identified bacterial species from *in silico* analysis of T-RFLP profiles. However it was noted that band 567 was only detected when band 155 was detected. In addition no species consistent with a predicted T-

RF of this length was identified by bacterial clone sequence analysis. Finally, it was also noted that a restriction fragment of length 567 was predicted fro the genetic sequence of the 16S gene in *P. aeruginosa*. Additional work was done to perform T-RFLP on pure cultures of *P. aeruginosa*. When this was performed, a band was detected at length 567, in addition to one at 155. This was taken as conclusive proof that the band seen in T-RFLP results at band length 567 was indeed a second fragment *P. aeruginosa*. Thus, when analysing results, percentage abundances at 155 were combined with percentage abundance at 567 to derive the final percentage abundance for *P. aeruginosa*.

2.3.3 16S Ribosomal Clone Sequencing

Clone library construction and sequencing were carried out at The Wellcome Trust Sanger Institute (The Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK) as described previously (Ludwig *et al.* 2004). One hundred and ninety two colonies of *E. coli,* with 16S sequence inserts from original bacteria, were randomly selected for sequencing from agar plates for each patient. Sequences were aligned using the 'NAST aligner' (Ashelford *et al.* 2006) and these alignments were subject to extensive manual curation using the ARB package (DeSantis *et al.* 2006) before further analysis. Sequences were tested for chimeras with Mallard (Ashelford *et al.* 2005) Bellerophon at Greengenes (Wang *et al.* 2007) and Pintail (Schloss & Handelsman 2005) and any sequence that appeared to be chimeric were removed. After removal of chimeras and other suspect sequences, the remaining sequences (deposited in Genbank under accession numbers FM995625-FM997761) were initially given a broad classification to the phylum level using the Classifier tool at the RDPII website (Zhang *et al.* 2000). To obtain more detailed taxonomic information, the

sequences were divided into phylotypes. Distance matrices were then entered into the DOTUR program (Schloss & Handelsman 2005) set to the furthest neighbour and 99%-similarity setting. The resulting phylotypes were then assigned similarities to nearest neighbours using MegaBLAST (Kelly *et al.* 2002). The Shannon diversity index (SDI) for each individual patient sample was calculated using DOTUR (Hewson *et al.* 2006).

2.4 Sputum Inflammatory Mediators

2.4.1 Clinical Sampling Methods

The effect of delays in processing samples on inflammatory mediators is not known (Health Protection Agency 2004). In order to minimise the time from sputum production to processing, samples for measurement of inflammatory mediators were only obtained when the sample could be frozen at -80^{o}C within 2 hours. In general, this meant that these samples were expectorated by participants whilst on site at Southampton General Hospital. Thus, samples expectorated by participants for microbial analysis whilst at home were invariably unsuitable, as transportation time by courier to SGH was greater than 2 hours.

Participants expectorated sputum samples for inflammatory mediator analysis whenever they attended SGH for other reasons. Examples of these reasons included routine outpatient appointments, visits to the Adult CF Unit for the start or end of a course of intravenous antibiotics or visits for annual review. Samples were expectorated into a 60 ml sterile container and then immediately frozen at -80^{o}C for further processing later. These samples were stored for between 1 week and 18 months before completion of processing.

2.4.2 Sputum Processing to Generate a Soluble Phase

Whole sputum samples were thawed on ice in batches. Adherent saliva was removed from the mucus component of the sample by sterile pipette and a sterile metal implement with the assistance of gravity. Saliva is more watery than mucus and samples were thawed on a slight incline to assist in the separation of saliva from sputum.

Up to 750mg of the sputum component was transferred to a 1.5ml Eppendorf tube and weighed. PBS was added in a 1ml:1mg ratio. Thus no more than 750mg of sputum could be added due to the size of the tube. Samples were then vortexed thoroughly on a Whirlymixer for 30 seconds, before being mixed on an orbital plate mixer for 10 minutes on ice. They were then vortexed again on the Whirlymixer for another 30 seconds. Each sample was visually assessed to ensure adequate homogenisation. Additional mixing was done if necessary. Once adequate homogenisation had been obtained, samples were centrifuged at 20,000g for 20 minutes at 4 ^{o}C. The resulting supernatant was aspirated using a pipette into 0.5ml Eppendorf tubes. The first and second tubes contained 20µL each. Additional tubes were filled with 100µL each. Finally a tube with any remaining supernatant was filled.

The first 20µL was used to measure neutrophil elastase (NE) activity on the same day. The additional tubes containing supernatant were frozen at 80^{o}C for measurement of inflammatory mediators at a later date.

2.4.3 Inflammatory Mediator Analysis

2.4.3.1 *Neutrophil Elastase*

Neutrophil elastase activity was measured in samples of CF sputum supernatant. Measurements were undertaken on the same day as the

supernatant was generated. Sputum supernatant samples were diluted in assay buffer (0.3M TRIS-HCl, containing 1.5M NaCl, pH 8.0). Samples were diluted to 1:1, 1:5 and 1:10, also 1:50 when required. To generate a standard curve, NE standard (human leucocyte elastase; Sigma Poole, UK) was used in serial dilutions. The diluted supernatant samples, and the NE serial dilutions of the NE standard were placed in the wells of a 96 well plate and allowed to pre-incubate for one minute at 37^{o}C . To each well was then added 90 μL of substrate (see below for method for substrate). The plate was allowed to incubate for a further 10 minutes at 37^{o}C. The colour change was read as an increase in light absorbance at a wavelength of 410 nm, using a microtitre plate reader (Dynex Revelation 4.21, Dynex Technologies, Worthing U.K.). Results from samples were interpolated from the standard curve, and adjusted to correct for the dilution factor to generate a result. Results were reported as mu of NE activity/mL (mu/mL).

Substrate was made as follows: N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide was as a concentrated stock solution in dimethylsulphoxide (DMSO) at 88.9mM (i.e. 10 mg/190 μL) and stored in 20 μL aliquots at –20^{o}C. On the day of assay, aliquots were diluted to 0.555 mM in assay buffer, as required.

2.4.3.2 *Interleukin-8*

Human IL-8 (huIL-8) was measured in all generated supernatant using PeliKine Compact™ human IL-8 ELISA kit [Sanquin Reagents, Amersterdam, The Netherlands]. This is an immunoassay of the 'sandwich-type'. In brief, a monoclonal anti-human IL8 antibody (huIL-8) is bound onto microtitre wells. HuIL-8 present in a standardised volume of supernatant or standard, is captured by the anti-HuIL-8antibody. Non-bound IL-8 is removed by washing. Following this, biotinylated sheep anti-huIL-8 antibody is added, which binds to

the bound huIL-8 from the supernatant. Excess biotinylated sheep anti-huIL-8 is washed away, followed by addition of horseradish peroxidise (HRP) streptavidin, which binds on the biotinylated side of the anti-huIL-8 sandwich. A substrate solution is then added that generates a yellow coloured product in proportion to the amount of huIL-8 in the supernatant or standard. The reaction is then terminated by a stop solution, and absorbance is then measured in a microtitre plate reader. The wells containing the standard solution allow for the creation of a standard curve, and the concentration of IL-8 in the wells containing supernatant is determined by interpolation of the absorbance with the standard curve.

Assay Procedure

PeliKine Compact™ human IL-8 ELISA kit instructions were followed at all times.

Day One: Step One - Preparation of Samples and Reagents

On the day before the main assay day, the coating buffer was prepared. This consisted of 0.1M Carbonate/bicarbonate solution with ph 9.6. This was prepared by making two solutions, A and B. Solution A was made from 1,24 g $Na_2CO_3.H_20$ in 100 ml distilled water. Solution B was made by adding 1.68 g of $NaHCO_3$ in 200 ml of distilled water. Solution B was added to solution A until a pH of 9.6 was reached. The coating antibody (a monoclonal anti-huIL-8 antibody) solution was diluted 1:100 with the coating buffer. One hundred microlitres of this solution was added to each well of the 96 well plate. The plate was then incubated at room temperature overnight.

Day Two: Step Two - Preparation

The morning of the assay a number of buffer solutions were prepared. A stock solution of phosphate buffered saline (PBS) (Ca^{2+} and Cl^- deplete) was prepared. A polysorbate surfactant washing buffer was prepared by adding 50µL of TWEEN to 1000ml of PBS. Blocking buffer was prepared by adding 500µL of the pre-prepared blocking reagent to 25 mL. Dilution buffer was prepared by diluting the provided 5-fold concentrated dilution buffer with distilled water to provide a working strength dilution buffer.

Step Three – Washing

The contents of the wells were discarded. Each well was then filled with wash solution before being discarded again. Complete removal of the wash solution was achieved by tapping. This wash process was repeated until each well had been washed five times. After the final aspiration the wells were dry.

Step Four – Blocking Procedure

To each of the wells of the microtitre plates was added 200µL of blocking buffer (prepared as described above). The plate was then covered with an adhesive seal and gently agitated, by tapping the edge of the plate a few times, to ensure adequate mixing of the contents of the wells. The plate was then allowed to incubate at room temperature for one hour. After incubation the wells were again washed 5 times, following the procedure described in Step Three.

Step Five – Preparation of IL-8 Standard

A natural human IL-8 standard has previously been calibrated against the World Health Organisation (WHO) Interim International al Standard (IL-8 89/520; National Institute for Biological Standards and Control, Potters Bar, U.K. 1 WHO unit = 10 ng IL-8). Using this standard, serial dilutions of IL-8 were prepared to

allow the calculation of a standard curve with which to compare the study sample supernatant results with. The seven serial dilutions of Il-8 contained 240, 96, 38.4, 15.4, 6.1, 2.5, and 1 pg/mL respectively, with a blank well representing 0 ng/mL.

Step Six – First Incubation

In duplicate, 100 µL of the prepared samples and standards were transferred to the appropriate wells. The plate was then covered and agitated gentle by hand for a few seconds. The plate was then incubated at room temperature for one hour. After one hour the supernatant was aspirated from the wells and the plates was washed repeatedly as outlined in Step Three (above).

Step Seven – Second Incubation

Just prior to use, the concentrated biotinylated IL-8 antibody was diluted by adding 120 µL to 12mL of working strength dilution buffer. All except the substrate blank wells had 100 µL of the diluted biotinylated antibody solution added. The plate was again covered, and agitated by hand for a few seconds, before being left to incubate at room temperature for one hour. After one hour the supernatant was aspirated from the wells and the plates was washed repeatedly as outlined in Step Three (above).

Step Seven – Third Incubation

Just prior to use, 3 µL of streptavidin-HRP conjugate was added to 30 mL of dilution buffer to make working strength streptavidin-HRP conjugate. The substrate blank wells were left empty, but 100 µL of working strength streptavidin-HRP conjugate was added to all other wells. The plate was again covered, and agitated by hand for a few seconds, before being left to incubate

at room temperature for 30 minutes. After thirty minutes the supernatant was aspirated from the wells and the plates was washed repeatedly as outlined in Step Three (above).

Step Seven – Fourth Incubation

Approximately ten minutes prior to its use, the substrate buffer was prepared in the following way: 15.0 g of sodium acetate ($CH_3COONa.3H_2O$) was dissolved in 800 mL of distilled water. The pH was adjusted to 5.5 with glacial acetic acid, and the final volume was made up to 1 litre with distilled water. To all wells, including the substrate blank wells, was added 100 µL of substrate solution. The plate was again covered, and then gently agitated by hand for a few seconds. The plate was then incubated at room temperature in the dark for thirty minutes.

Step Eight – Stop Enzymatic Reaction

Sulphuric acid (1.8 M H_2SO_4 solution in distilled water) was added in 100 µL aliquots to all wells.

Step Nine – Plate Reading

Within 30 minutes of the stop reaction, the enzymatic colour change in each of the wells of the plate was read by a microtitre plate reader (Dynex Revelation 4.21, Dynex Technologies, Worthing U.K.). Sample results were interpolated from the generated standard curve. Results were reported in ng.

2.4.3.3 *Eosinophil Cationic Protein*

Eosinophil Cationic Protein (ECP) was measured in sputum supernatant that was generated using the protocol described in detail above. ECP was

measured using a commercially available kit (Medical and Biological Laboratories International Corporation, Woburn, USA). The manufacturer's instructions were followed at all times. In brief this assay is a sandwich ELISA which measures ECP with a minimum detection limit of 0.125 ng/mL and does not cross react with other eosinphil derived products such as Eosinophil derived neurotoxin (EDN).

Preparation of Reagents and Samples

All reagents were brought up to laboratory room temperature (20-25^{o}C) prior to use. The wash solution was prepared by diluting 100 mL with 900 mL of distilled water. Sputum supernatant samples for analysis, that had been stored at -80^{o}C, were thawed and brought up to laboratory room temperature. They were then diluted with the assay diluent (0.09% sodium azide with 1% Goat serum preservative) to a final concentration of 1:5, 1:50 or 1:500. The positive control was also sequentially diluted with the diluent, leaving eight aliquots.

Assay Procedure:

Step One – Sample Incubation

Aliquots of 150µL of the prepared samples and standards were added to a 96-well polyvinyl preparation plate. Then 100 µL of each sample was transferred to the antibody coated microwell and mixed well. Incubation at room temperature was allowed for 60 minutes.

Step Two - Washing

The contents of the wells were discarded. Each well was then filled with wash solution before being discarded again. Complete removal of the wash solution

was achieved by tapping. This wash process was repeated until each well had been washed four times.

Step Three – Conjugate incubation

One hundred microlitres of conjugate reagent (Horseradish peroxidase conjugated anti-human ECP polyclonal antibody, 1% Bovine Serum Albumin (BSA) was pipetted into each of the wells and incubated at room temperature for 60 minutes. This was followed by further washing as detailed in Step Two (above).

Step Four – Substrate Incubation

After washing, 100µL of substrate reagent (3,3',5,5'-Tetramethylbenzadine (TMB) with hydrogen peroxide (H_2O_2)) was added to each well and incubated at room temperature for 10 minutes.

Step Five – Stopping Reaction

After 10 minutes, 100 µL of stopping reagent (0.5 mol/L sulphuric acid (H_2SO_4)) was pipetted into each well. Reading of the plates took place within 30 minutes of stopping solution having been added.

Step Six – Reading

All plate reading took place using a computer spectrophotometer (Dynex Revelation 4.21, Dynex Technologies, Worthing U.K.) at wavelength of 450 nm.

Step Seven – Calculation of Results

The wells that contained the standard solution allowed for the creation of a standard curve. The concentration of ECP in the wells that contained

supernatant was determined by interpolation of the absorbance with the standard curve. The final concentration was determined by multiplying the result by the dilution factor (e.g. x5, x 50 or x500 – see Preparation of Reagent and Samples above). Results were reported in ng/mL.

2.5 Definitions

Bacterial community stability: a community of bacteria which retain the same dominant species, and for which all the species present at >10% remain in the same rank abundance position.

Subjects: shall be used to refer to any particular individual who took part in this study. When referring to a group of participants the lower case 'subject(s)' shall be used.

Clinical stability: at 30 days before the start and 30 days after the end of systemic antibiotics for changes in pulmonary symptoms.

'***Species Richness***

In order to describe the species richness of a sample or population, the number of species was counted (species richness). Bacterial species richness measures a similar quality of a bacterial community to the Slope of the rank abundance curve (see below). However, unlike Slope, species richness has the advantage of giving a valid score for communities consisting of only one species. Variations in species richness within subjects (temporal variations) and between subjects (spatial variations) are described. In addition, as not every species was found in every sample, a measure of the cumulative species richness was used. Subjects for whom cumulative richness became asymptotic quicker than others could be thought of has having a more stable bacterial

community that those subjects for who the cumulative species abundance curve took longer to reach a plateau.

Bacterial Community Composition

In order to describe the similarity between different samples, a number of measures were used. Sorensen's Index of Similarity was used (Pye *et al.* 2007). The formula for Sorensen's Index is 2W/(a1 + a2); where 'a1' is the total number of species detected in sample 1, and 'a2' is the total number of species detected in sample 2, and W is the number of species that are common to both samples. It is the same measure as the Dice Co-efficient. It is used for comparing detection of species between samples were the presence or absence of species are presented in a binary format. In the form described it cannot be used for data that is in a quantitative or semi-quantitative format. It always gives a value in the range 0-1.

In addition to the Sorensen Index of Similarity, an additional measure of similarity that took in to account relative abundance was used. The Bray-Curtis Index was used, due to its proven validity and acceptance (Magurran 2003). In addition, its use was familiar to the research team.

Bacterial Community Structure

In an effort to measure species evenness in a given sample, additional measures of community structure were used. In order to convert the species dominance curves to analysable values, a variety of measures were derived. Firstly, the slope of plot of the log(*n*) of the ranked percentage values was used, termed the slope of the rank abundance curve (Ager *et al.* 2009). The more negative the value the steeper the species dominance curve, and the less even the species distribution. A minimum of two values were required to calculate a

slope. For a number of samples there was only one species detected (i.e. value = 100%) and thus no slope could be calculated. Such samples were excluded from this analysis.

In addition to the described measures on individual sputum samples, a number of multivariate methods were used in an attempt to determine patterns in large data sets. Principle Component Analysis (PCA) and Multidimensional Scaling (MDS) were used. Results were found to be similar, and thus results of PCA are presented, as this was felt to be a more robust technique. Multivariate analysis and graphical output were undertaken using Community Analysis Package v.4 (www.pisces-conservation.com).

Core/Satellite Analysis

Three subjects had samples analysed from times other than before, during and after one single CFPE: Subjects 02, 09 and 14, who had 45, 121 and 59 samples analysed respectively. When assessing for core/satellite differentiation, the greater the number of samples the more robust the analysis (Magurran 2003). Only the sample size for Subject 09 was sufficiently large to adequately differentiate between core and satellite species.

2.6 Statistical Analysis

All data were checked visually for outlying values that might represent erroneous values. This was performed with the naked eye, histograms and box and whisker plots. Once identified, such values were rechecked against source data wherever possible. Also using histograms, the data were assessed for normality of distribution. Data that conformed to a normal distribution were

analysed with parametric statistical tests, whist those which did not conform to a normal distribution were analysed with non-parametric tests.

The majority of the statistical data analysis was undertaken using SPSS (v. 16 for Windows; SPSS, Chicago, Illinois, USA) or Microsoft Office Excel for Windows 2003. Graphical display was generated with SPSS, or Microsoft Office Excel. The Principle Component Analysis was performed using Community Analysis Package 4 2007 (Pisces Conservation Ltd., Pennington, Hampshire, UK).

2.7 Validation of Study Techniques

2.7.1 Visual Analogue Scales Repeatability Testing

Results for repeatability testing for the VASs are presented in Table 2-2 below. All four VAS domains performed extremely well, with very high measures of repeatability, assessed by both Cronbach's Alpha, and by Intraclass Correlation Coefficient (ICC). General Wellbeing performed best, with a Cronbach's Alpha of 0.998, and ICC of 0.991 (0.962-0.999). Although still performing well, cough VAS performed the worst, with a Cronbach's Alpha of 0.989, and an ICC of 0.944 (0.791-0.996).

	Subjects (& Repeats) N	Cronbach's Alpha (0-1)	Intraclass Correlation Coefficient (0-1)	95% Confidence Limit	
				Lower	Upper
Breathlessness VAS	4 (5)	0.998	0.988	0.948	0.999
Cough VAS	4 (5)	0.989	0.946	0.791	0.996
Sputum VAS	4 (5)	0.997	0.987	0.944	0.999
GWB VAS	4 (5)	0.998	0.991	0.962	0.999

Table 2-2 Measures of Repeatability Testing for VASs

2.7.2 Sample Delivery Cold Chain

As outlined above in 2.1.5.3.2, the ability of the cold chain to deliver samples to the laboratory from subjects in the community, often many miles away, was audited. Results are presented in Table 2-1 below. An acceptable temperature was defined as $\geq 0^{o}C$, and $\leq 6.0^{o}C$. These values were chosen by the research team, as the UK HPA SOP makes no reference to the optimal temperature to store samples prior to processing, except to say that refrigeration is preferable to storage at ambient temperature (Health Protection Agency 2004). Overall

the cold chain was felt to offer an acceptable service. The 14 deliveries presented here had a mean time from being produced to being unpackaged in the laboratory of 831 minutes, or just under 14 hours. There was a mean time to achieve adequate cooling of 91 minutes. All subjects had a mean temperature (once cooling had been achieved) within the target range, with the lowest mean being 0.08 in subject 10, and the highest being 5.35 in subject13. Only one of the 14 subjects had a median value outside the target range (subject 01), along with a value of 89% of the time spent outside the target range (after cooling was achieved). This appears to have been caused by a prolonged period of time at 7.0^{o}C, reflected by the maximum temperature in this case being 7.5^{o}C. The month that the study was performed in seemed to have no bearing on the mean temperature.

	Subject Number														Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Total Time (min)	302	210	1090	1068	322	1428	364	1496	216	372	1316	1090	594	1770	831
Time to stabilise (min)	34	72	42	42	36	496	42	32	18	14	288	70	18	66	91
Time above 6°C	270	0	0	0	30	0	0	136	0	0	0	0	134	0	41
Time below 0°C	0	0	244	10	0	44	244	22	0	136	4	244	0	0	68
Total time out of range	270	0	244	10	30	44	244	158	0	136	4	244	134	0	108
Percentage of time out of range	89	0	22	1	9	3	67	11	0	37	0	22	23	0	20
Min temp	1.5	2.5	-3.5	-0.5	1.5	-2	-1.5	-8	0	-3.5	-1.5	-9	4	0.5	-8
Max Min	7.5	5.5	5.5	5.5	9	4.5	5.5	16	6	5	5.5	6	9	6	9
Mean Temp	5.34	3.44	1.65	1.71	5.17	1.38	1.91	4.01	3.15	0.08	3.41	1.66	5.35	3.30	
Median Temp	6.5	3.5	2.5	1.5	4.5	1.5	1	3.5	3.5	0.5	3.5	2.5	4.5	3.5	
SD	2.06	0.76	2.24	0.58	1.79	0.94	0.187	2.52	1.9	1.95	1.22	2.24	1.29	0.494	
Month of study	Oct	Oct	Nov	Dec	Oct	Dec	Nov	Jan	Jan	Feb	Feb	Mar	Mar	Oct	

Table 2-3 Results of Specimen Delivery Cold Chain Auditing

2.7.3 Duration of Transportation of Sample to Laboratory

In order to assess whether there was delay from samples being expectorated to there arrival and subsequent processing in the laboratory, a random selection of clinical monitoring forms were analysed. After using SPSS to select 2% of samples at random, the time of expectoration and the time of delivery to the laboratory were documented. A summary of results are shown in Table 2-4 below. Of the 35 samples selected for analysis, four did not have the delivery time recorded. For the other 31 samples, the median time to delivery of samples was 9.8 hours, with a mean of 12.6 hours (SD±9.4). The maximum time to delivery was 30.2 hours.

After arrival of samples in the laboratory samples were processed for bacterial culture on selective media plates. This resulted in a period of time, after delivery but before samples were placed in the -80^{o}C freezer. This delay was audited internally by senior staff within the HPA. Results are presented below in Table 2-5 below. The mean time to freezer from arrival in the laboratory was 42 minutes, with a maximum time of 105 minutes.

Overall these data suggest that the delivery and cold chain worked satisfactorily to ensure the safe receipt of appropriately chilled samples in good time.

Number	Valid	31.0
	Missing	4.0
Mean (hours)		12.6
Median (hours)		9.8
Std. Deviation (hours)		9.4
Minimum (hours)		1.6
Maximum (hours)		30.2
Percentiles (hours)	25	3.6
	50	9.8
	75	22.8

Table 2-4 Results of time to delivery audit

Number	Valid	12
	Missing Data	0
Mean (minutes)		42
Median (minutes)		35
Std. Deviation (minutes)		30
Maximum (minutes)		105
25-75th Percentiles		26-48

Table 2-5 Results of time to freezer analysis

2.7.4 Reproducibility, and Effects of Delays in Time to Freezer on Bacterial Diversity

As part of the validation process, a small pilot study was undertaken on sputum samples to assess the effects of time spent in the refrigerator prior to freezing on measures of bacterial diversity. Expectorated samples were collected and immediately homogenised prior to separating in to separate aliquots. Each aliquot was stored at 4^{o}C for different periods of time.

The graphs below (

Figure 2-2) show that there is little effect of refrigeration time prior to freezing of samples up to 36 hours. At 72 hours, the next time period assessed, there were detectable differences in the bacterial community, mainly reflected by the dominant species becoming less dominant. Such results confirmed the validity of both the study protocol and the extensive efforts in establishing a reliable and efficient cold chain to deliver samples from subjects in the community. In addition it provided validation of the process of T-RFLP, as the whole process, from expectoration to PCR and T-RFLP had to be working with problem to achieve these highly reproducible results.

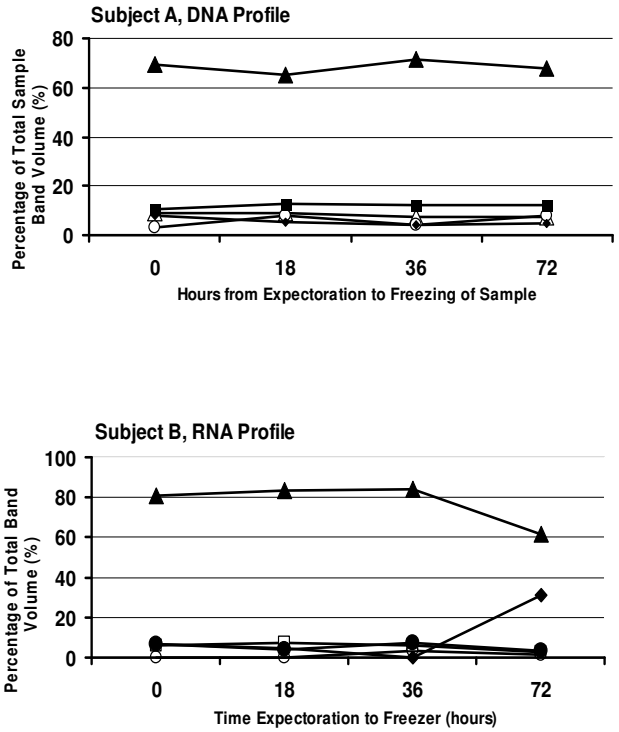

Figure 2-2 Effects of Time from expectoration to sample freezing for two subjects (A and B).

2.7.5 Repeatability of Molecular Techniques

In addition to demonstrating the effects of increasing time of storage at 4^{o}C,

Figure 2-2 above also demonstrates the reproducibility of the whole process of T-RFLP. If there were methodological flaws, it would be expected that significant differences in the bacterial populations would be seen with each different sample processed. It can be seen that this was not the case. This suggests that T-RFLP, as undertaken during this study, is reproducible, as has been previously described (Rogers *et al.* 2004). In turn, this gives confidence that any changes in the bacterial community detected over time during the rest of the study were due to other reasons than methodological ones.

2.7.6 Discussion

Others have assessed the effects of temperature and delays on bacteria in sputum samples (Pye *et al.* 2007), but only using culture. This previous study by Pye *et al.* was aimed at demonstrating whether sputum samples could be reliable analysed after being sent by post (at ambient air temperature ~20^{o}C) to the laboratory by the subject. This paper was published after the work presented here was completed, and did not make clear how long the delay from sample expectoration to analysis was. By culture, they found that transportation at ambient temperature provided results that were closer to results of immediate analysis than were results from samples that had been refrigerated. However, it adds weight to the assertion that samples arriving by courier were in the best possible condition for analysis. The results of assessment of possible delays in samples reaching the laboratory provided additional reassurance that samples were arriving in a timely manner. The absolute minimal delay possible would provide the optimum quality samples, and a mean of 13 ½ hours from being produced in the community for samples to reach the laboratory and processed is a satisfactory result.

Chapter 3 - Results Overview

3.1 Overview

Ethical approval was obtained for the study for the Southampton and Southwest Hampshire Ethics Committee (LREC 06/Q1704/26). Fourteen subjects agreed to participate in the longitudinal study after providing written informed consent. Subjects commenced in the study between 1st June 2006 and 5th October 2006. No subjects withdrew, and all continued in the study for at least 12 months. The last subject left the study on 4th October 2007.

3.1.1 Subject Demographics

Subject Baseline Characteristics

The subjects were selected by review of the notes of patients in the adult CF service at SGH, and in discussion with the adult CF clinical team.

The 14 subjects had a mean age of 30.2 years (SD $\pm$ 12.0; range 17.9 – 55.7 years). They had a mean FEV1 of 1.79 L (SD $\pm$ 0.71 L; range 3.21 – 0.73 L), and a mean FEV1% predicted of 53.3% (SD $\pm$ 18.9%; range 17.4 – 86.4%). In terms of pulmonary disease severity classification, based on the Cystic Fibrosis Foundation disease severity classification (FEV1% predicted: normal lung function ≥ 90%; mild pulmonary impairment 70-89%; moderate pulmonary impairment 40-69%; severe pulmonary impairment < 40%) no subjects had normal lung function, 3/14 had mild impairment, 7/14 had moderate pulmonary impairment, and 4 had severely impaired pulmonary function. They had a mean body mass index (BMI) of 21.9 kgm^2 (SD $\pm$ 3.75; range 18.0 – 29.0) which was within the accepted normal range for healthy adults. Twelve of fourteen

subjects had CF related pancreatic insufficiency; eight of fourteen had CF related diabetes (CFRD). There were six females and eight males; their lung function by gender was 58.1%(±18.4%) and 49.6% (±19.7%) respectively; difference *p*=ns. Further details of baseline characteristics for individual subjects can be found in Table 3-1and Table 3-2 below.

Subject No	Sex	FEV1 (L)	FEV1 % Predicted	Disease severity.	BMI (kgm^2)	Age	P.S.?	CFRD?	Genotype
1	M	2.51	60.9	1	29.0	30.7	Yes	No	ΔF508/NK
2	F	1.3	57.0	2	18.2	45.2	No	Yes	ΔF508/ΔF508
3	M	1.23	38.0	3	19.9	46.9	No	Yes	ΔF508/NK
4	F	1.3	42.0	2	27.1	29.9	Yes	No	ΔF508/T20
5	F	1.23	39.3	3	18.0	22.8	No	Yes	ΔF508/ΔF508
6	M	1.59	48.7	2	23.9	55.7	No	Yes	ΔF508/T20
7	F	1.68	51.0	2	20.3	21.1	No	No	ΔF508/T20
8	M	1.91	60.8	1	19.4	40.4	No	Yes	ΔF508/ΔF508
9	M	0.73	17.4	3	18.4	22.6	No	Yes	ΔF508/ΔF508
10	F	2.86	86.4	0	22.5	17.9	No	No	ΔF508/ΔF508
11	F	2.17	73.0	1	21.0	24.8	No	No	ΔF508/T20
12	M	1.19	30.0	3	20.4	20.5	No	Yes	ΔF508/ΔF508
13	M	2.1	66.0	1	28.5	20.5	No	No	ΔF508/ΔF508
14	M	3.21	75.0	1	21.1	23.6	No	Yes	ΔF508/ΔF508

Table 3-1 Baseline characteristics for individual subjects. FEV1 = Forced Expiratory Volume in the first second in Litres; FEV1% Predicted = FEV1 as a percentage of the predicted value for a person of the same age, height, gender and ethnicity; BMI = body mass index; P.S. = Pancreatic sufficient; CFRD = CF related Diabetes Mellitus.

	FEV1 (L)	FEV1 (% Predicted)	BMI (kgm2)	Age (years)
N	14	14	14	14
Mean	1.79	53.25	21.98	30.17
Median	1.64	54.01	20.70	24.18
Std. Deviation	0.71	18.93	3.75	11.97
Minimum	.73	17.4	18.0	17.9
Maximum	3.21	86.4	29.0	55.7
Interquartile Range	1.23-2.26	39.0-67.8	19.2-24.7	21.0-41.6

Table 3-2 Baseline characteristics for all subjects

Values for FEV1, BMI and age did not conform to a normal distribution. Further statistically analysis that involved these variables was performed using non-parametric techniques. Values for FEV1 % predicted did conform to a normal distribution, and statistical analysis that only involved this, and other normally distributed data, were analysed using parametric methods. Histograms for values demonstrating these distributions can be found in the appendix (9.2 below)

Subject Baseline Medications

As would be expected, no two subjects were on the same medications at baseline.

Thirteen of the fourteen subjects were taking a long term antimicrobial therapy, with 11 taking a macrolide and 12 taking a nebulised antibiotic. Two were on long term antifungal therapy in the form of oral itraconazole.

Ten subjects were on mucolytic therapy, either in the form of dornase alpha (a DNAse), or inhaled hypertonic saline. Thirteen of fourteen were on inhaled corticosteroids, with only one taking oral corticosteroids. This subject was also on long term itraconazole for Allergic Bronchopulmonary Aspergillosis (ABPA), and had been demonstrated to have inadequate endogenous adrenal corticosteroid production.

		Subject Number													
	Long term Medication	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**	**12**	**13**	**14**	**Total**
Antimicrobial Therapy	**Macrolide**		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes			**11**
	Colomycin nebulised		Yes	Yes		Yes	Yes	Yes	Yes	Yes		Yes			Yes	**9**
	Tobramycin nebulised												Yes			**1**
	Tobramycin/Colomycin nebulised (alt. months)										Yes			Yes		**2**
	Itraconazole					Yes								Yes		**2**
Mucolytic	**DNAse**	Yes		Yes	Yes	Yes	Yes	Yes		Yes	Yes	Yes		Yes		**10**
	Hypertonic saline									Yes						**1**
Corticosteroids	**Oral corticosteroids**													Yes		**1**
	Inhaled corticosteroids	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes		Yes	Yes	**13**
Diabetic treatment			Yes	Yes					Yes	Yes					Yes	**5**

Table 3-3 Long term medications taken by subjects during study

3.2 Pulmonary Exacerbations

The 14 subjects had 42 physician defined pulmonary exacerbations necessitating treatment with antibiotics. The range was 0-5, with a median of 3. See Figure 3-1 below .

For these 42 pulmonary exacerbations, subjects required 30 courses of intravenous antibiotics for CFPEs and 21 courses of oral antibiotics (see Figure 3-2 below.

There were also two courses of elective intravenous antibiotics administered to two different patients. In addition one subject had a course of oral antibiotics for an unrelated skin infection. See Figure 3-1 and Figure 3-2 below.

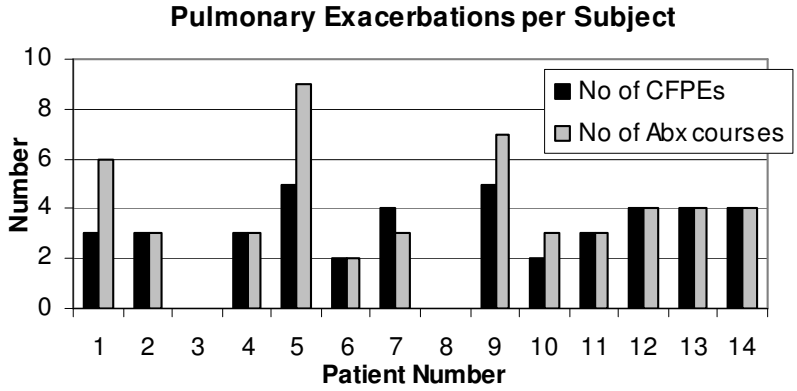

Figure 3-1 Pulmonary Exacerbations and Antibiotic Courses per Subject.

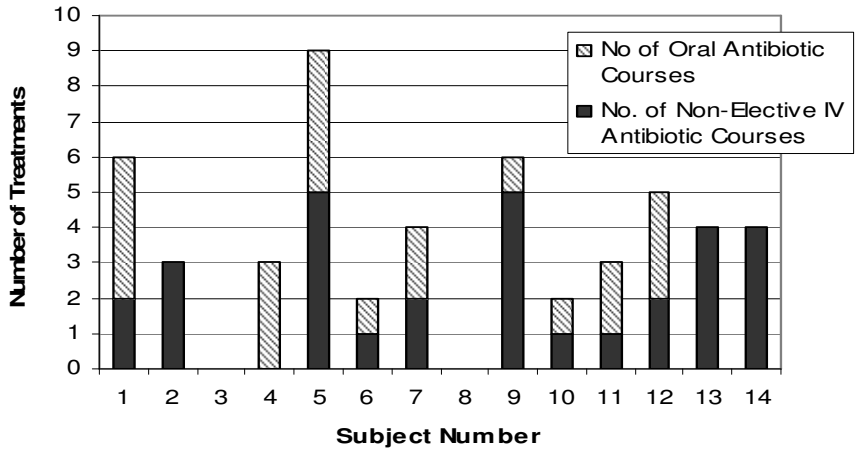

Figure 3-2 Type of Treatment for CF Pulmonary Exacerbations by Subject

Subject No.	No. of CFPEs per Subject	No. of Abx Courses for CFPEs	No. of Non-Elective IV Courses	No. of Oral Courses	No. of Elective IV Antibiotic Course	No. of Unrelated Abx
1	3	6	2	4	0	0
2	3	3	3	0	0	0
3	0	0	0	0	0	0
4	3	3	0	3	0	0
5	5	9	5	4	0	0
6	2	2	1	1	0	1
7	4	4	2	2	0	0
8	0	0	0	0	0	0
9	5	6	5	1	1	0
10	2	2	1	1	1	0
11	3	3	1	2	0	0
12	4	5	2	3	0	0
13	4	4	4	0	0	0
14	4	4	4	0	0	0
Total	**42**	**51**	**30**	**21**	**2**	**1**

Table 3-4 Pulmonary Exacerbations and antibiotics per subject

3.3 Results of Bacterial Culture

In total 2061 sputum samples were received in to the Health Protection Agency laboratory at Southampton General Hospital. The results of selective bacterial culture (as outlined in Section 2.2.1 above) are summarised in Figure 3-3.

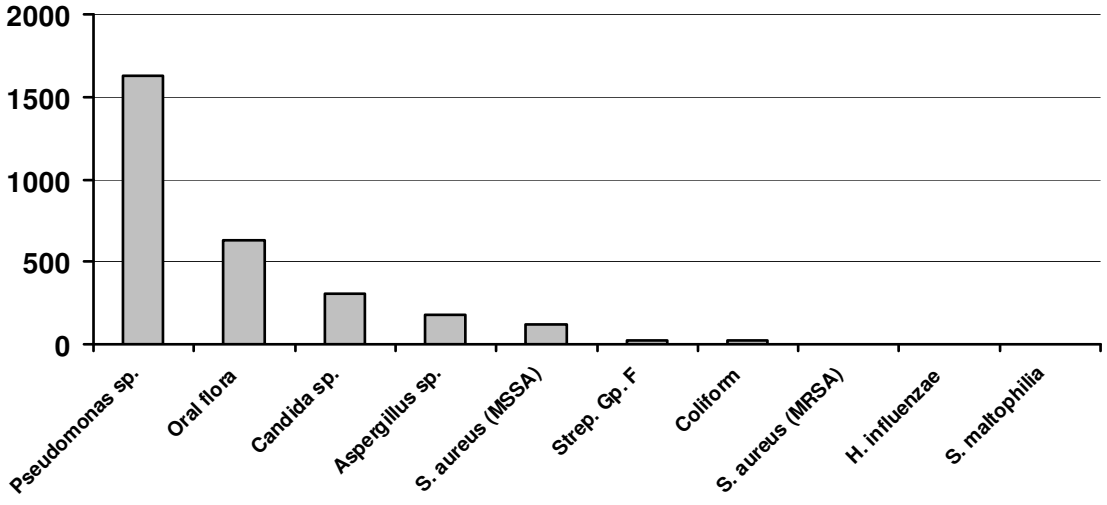

Figure 3-3 Summary of organisms detected by selective culture (all subjects).

It can be seen that, unsurprisingly, Pseudomonas species make up the most frequently detected species. 'Pseudomonas species' includes non-mucoid *P. aeruginosa* (n=301), mucoid *P. aeruginosa* (n=377), Pseudomonas species that were not typed (947) and *Pseudomonas fluorescens* (n=1). Pseudomonas species that were not typed are clinically assumed to be *P. aeruginosa*. Previous clinical surveillance work suggests that over 99% of these isolates are indeed *P. aeruginosa* (Mr. A. Tuck, HPA, Southampton General Hospital: personal communication). Oral flora include species such as group D β-haemolytic streptococci, α-haemolytic streptococci (non-pneumococcal), *S. sanguinis and mitis*, and coagulase negative (non-*S. aureus*) Staphylococci. Although anaerobic bacteria are a major component of normal oral flora (Kolenbrander 2000), the species identified under the heading 'Oral flora' do not include anaerobic species as culture was not performed in anaerobic conditions.

The third commonest bacterial group to be detected in this cohort of 14 was isolates of group F streptococci. This group is of interest, as it includes *S. constellatus* and related species that make up the *Streptococci milleri* group, which has previously been implicated as a possible cause of CFPEs by Sibley *et al.* (Sibley *et al.* 2008g).

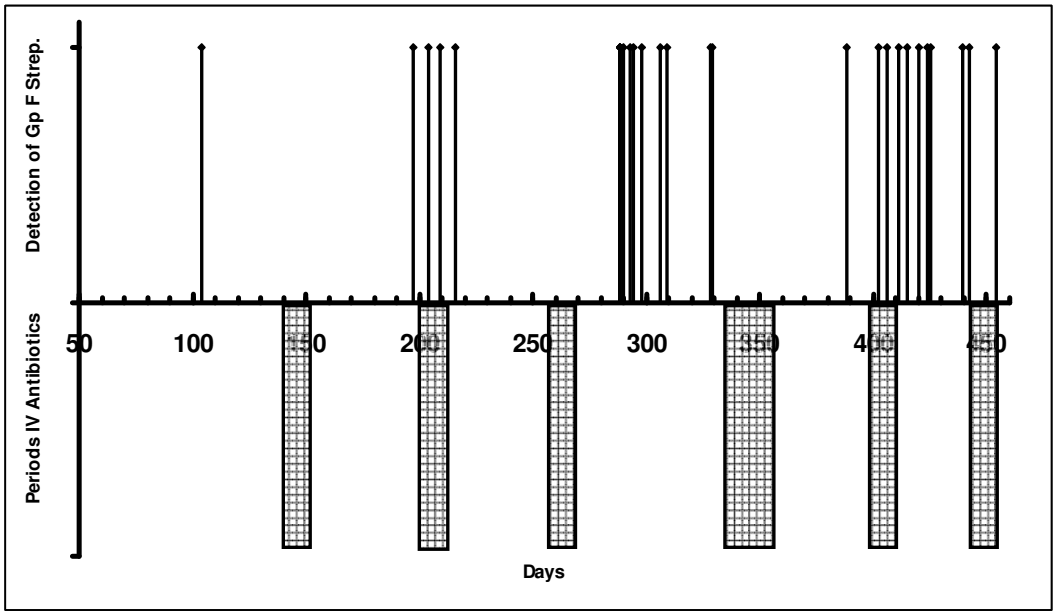

Figure 3-4 Relationship between CFPEs, antibiotics and detection of Gp F streptococci by culture, for Subject 09.

In Figure 3-4 above, is presented the data for the only subject (09) who cultured group F streptococci. It can be seen that there is no apparent relationship between the detection of these bacteria and either the occurrence of CFPEs, or with treatment with IV antibiotics. This data would not support a role for these bacteria alone to be a major cause of CFPEs, in contrast to the data from Sibley *et al.*. However it is noted that, although group F strep. species were detected, specific culture media optimised for the detection of bacteria from this group were not used in this study (Sibley *et al.* 2010).

Chapter 4 - Results: Clinical Monitoring

In total 2059 completed or partially completed Clinical Monitoring Forms (CMF) were received from the 14 subjects (mean 147 per subject SD±35.9). There were two outlying subjects: the minimum number of forms received for any subject was 52 (subject 04), the maximum was 209 (subject 09). The other 12 subjects produced between 121 and 174 CMFs during the study period. The variation in the number of forms received per subject depending on a number of factors, including frequency of sputum expectoration, frequency of exacerbation, holidays, and length of time in the study. For example subject 04 was unable to expectorate spontaneously as frequently as other study subjects, at an average of only one sample per week. In contrast, subject 09 could produce copious quantities of sputum per day, had 7 courses of antibiotics (with the associated daily sampling, compared to thrice weekly when not having a CFPE), had no holidays and agreed to remain in the study longer than 12 months.

4.1 Analysis of Individual Components of Clinical Monitoring

All variables measured using the Clinical Monitoring forms differed between subjects. The distribution of values for Visual Analogue Scales (VASs) and spirometric variables did not conform to a normal distribution, and thus data is presented as 'median' values and Interquartile range (IQR). In order to demonstrate the range and distribution of values for individuals, box and whisker plots were plotted for each VAS and spirometric category. The 'box' in each box and whisker plot represents the IQR, the horizontal line inside each box represents the median, the 'whiskers' represent ±1.5x IQR. Outlying values

between ±1.5 to 3x IQR are represented by '•', while extreme values greater than ±3x IQR are represented by '*'.

Table 4-1 below shows the distribution of values for each of the VASs. In general they performed similarly, with similar ranges, medians and IQRs. The General Wellbeing (GWB) VAS had the highest values for all measures.

	Br VAS	Cough VAS	Sputum VAS	GWB VAS
Mean (SD)	23.5 (16.8)	25.0 (17.7)	24.9(17.5)	27.2 (17.5)
Median	20	22	20	24
Min/Max	0/83	0/89	0/82	0/94
IQR	12-31	11-33	12-35	14-37

Table 4-1 Distribution of Values for components of Clinical Monitoring Form (subjects combined). All differences *p*=NS, by unpaired T Test. Br. = breathlessness; VAS = Visual Analogue Scale.

Different subjects showed variable patterns of correlation between their individual and summed VASs and lung function. Individual summaries are presented later. In order to provide an overview of the value of the Sum of VAS score that is presented along with FEV1 and FEV6 data in Response Profile Plots (RPP), a summary of the correlations is presented below in Table 4-2 below. Individual values are discussed in more detail in the following sections.

Subject		Correlation between:	
		Sum of VAS & FEV1	Sum of VAS & FEV6
1	rho	-.397**	-.448**
	p value	<.001	<.001
	N.	130	130
2	rho	-.486**	-.418**
	p value	<.001	<.001
	N.	146	145
3	rho	-.298**	-.210*
	p value	<.001	.015
	N.	135	135
4	rho	.204	.053
	p value	.151	.714
	N.	51	51
5	rho	-.484**	-.545**
	p value	<.001	<.001
	N.	139	139
6	rho	-.392**	-.390**
	p value	<.001	<.001
	N.	117	117
7	rho	-.522**	-.482**
	p value	<.001	<.001
	N.	159	159
8	rho	-.106	.084
	p value	.184	.293
	N.	158	158
9	rho	.500	.500
	p value	.667	.667
	N.	3	3
10	rho	.186*	-.260**
	p value	.020	.001
	N.	157	156
11	rho	-.315**	-.170*
	p value	<.001	.032
	N.	159	159
12	rho	-.084	-.129
	p value	.376	.172
	N.	113	113
13	rho	-.565**	-.329**
	p value	<.001	<.001
	N.	153	153
14	rho	-.437**	-.460**
	p value	<.001	<.001
	N.	171	171

Table 4-2 Summary of Correlations between Sum of VAS scores and lung function, by subject. Rho represents Spearman's Rank Correlation Coefficient; ** = statistically significant at p≤0.05 level.

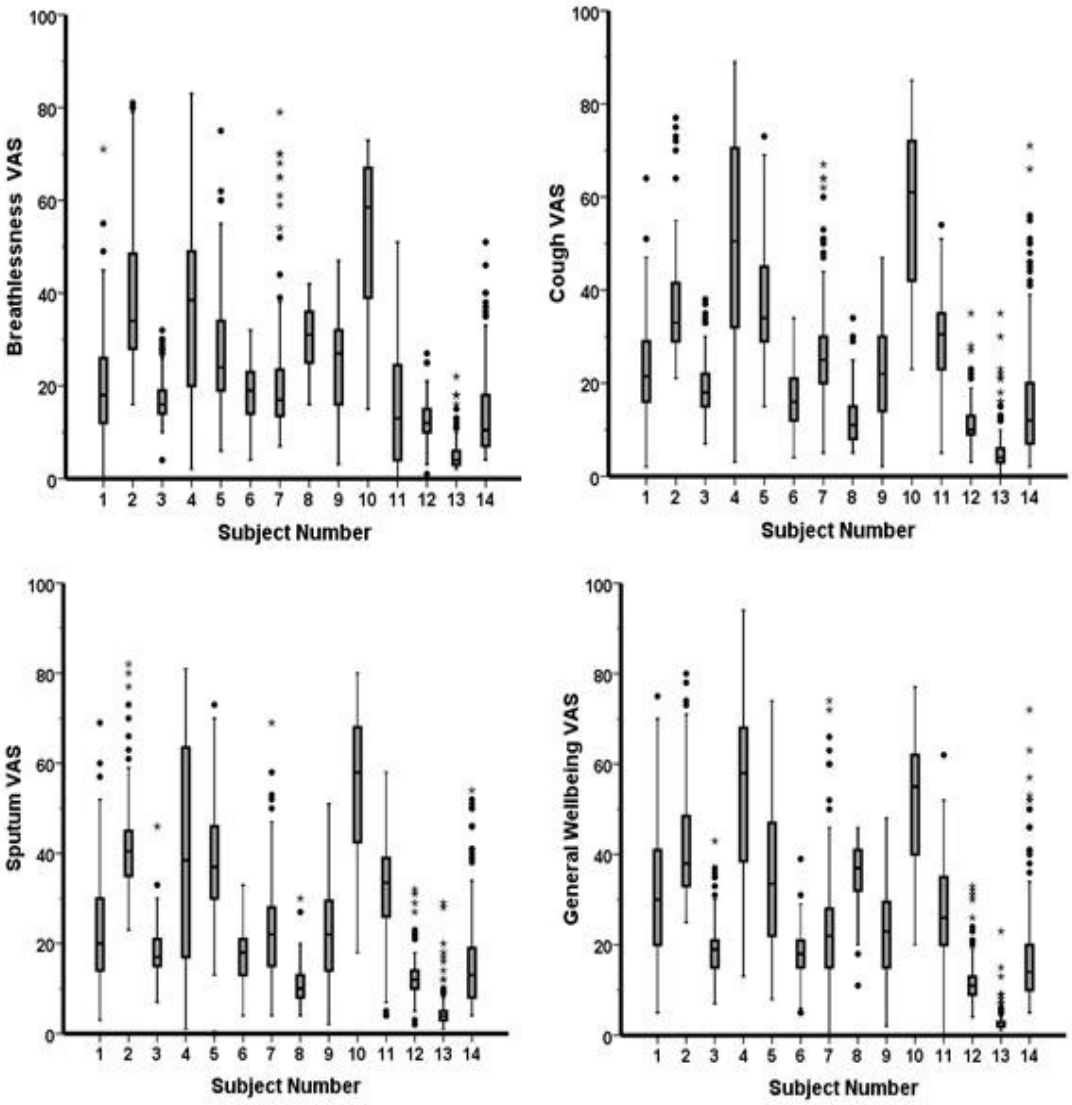

Figure 4-1 Visual Analogue Box and Whisker plots

4.1.1 Breathlessness VAS

Breathlessness VAS scores showed wide inter-and intra-subject variability. See Figure 4-1 above. Subject 04 showed the greatest intra-subject variability for Breathlessness VAS, with an IQR of 30, and a range of 81. In contrast subject 13 had an IQR of 3, and a range of 20, representing the lowest variability.

4.1.2 Cough VAS

Cough VAS scores showed wide inter-and intra-subject variability. See Figure 4-1 above. Subject 04 again showed the greatest intra-subject variability for Cough VAS, with an IQR of 38.75, and a range of 86. In contrast Subject 13 had an IQR of 3, and a range of 35, representing the lowest variability.

4.1.3 Sputum VAS

Sputum VAS scores showed wide inter-and intra-subject variability. See Figure 4-1above Subject 04 showed the greatest intra-subject variability for sputum symptom scores, with an IQR of 45, and a range of 82. In contract subject 13 had the lowest IQR (2) and a range of 28. Subject 08 also had low variability, with an IQR of 5, and a range of 26.

4.1.4 General Wellbeing VAS

GWB VAS scores showed wide inter-and intra-subject variability. See Figure 4-1 above. Subject 04 showed the greatest intra-subject variability for GWB symptoms, with an IQR of 29.75, and a range of 81. In contrast subject 13 had an IQR of 1, and a range of 22, representing the lowest variability.

4.1.5 Spirometric Values

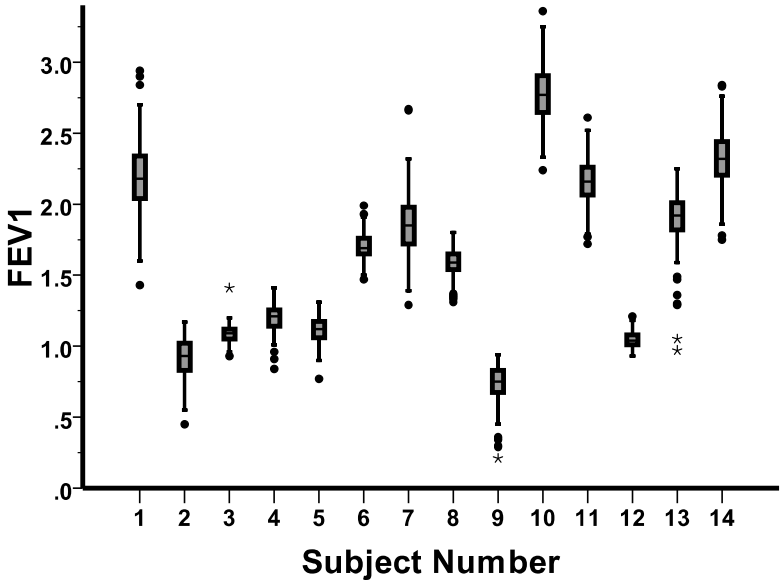

Figure 4-2 Forced expiratory volume in one second; box and whisker plot by subject.

Spirometric values at baseline showed a bi-modal distribution which persisted through the study (see Appendix Figure Figure 9-2). Group one (subjects 2, 3, 4, 5, 9, and 12) had more severe lung function with FEV1 values clustered around 1L. Group two (subjects 1, 6, 7, 8, 10, 11, 13, and 14), had less severe lung function, with FEV1 values clustered more widely around 2L. Subject 12 showed the least variability with an IQR of 0.06 L, and a range of 0.28 L. Subject 01 showed the greatest variability in FEV1 with an IQR of 0.3 L, and a range of 1.51 L. See Figure 4-2 above. A similar pattern was seen for FEV6 (forced expiratory volume in 6 seconds). A box and whisker plot of the values for FEV6 is presented in the Appendix. See Figure 9-4 below.

FEV1 and FEV6 values were not analysed for correlation as they are not independent variables, as FEV1 is an integral independent component of FEV6.

4.1.6 Correlations between components of Clinical Monitoring Form

In order to asses the validity of the domains chosen for the symptom components of the Clinical monitoring Form, it was necessary to correlate the individual domains with each other. In Figure 4-3 below, it can be seen that there are strong linear correlations between all combinations of symptoms (R^2 range 0.55 – 0.858). Overall the strongest correlation is between 'Cough' and 'Sputum'; the weakest correlation is between 'Sputum' and 'Breathlessness'. For 'General Wellbeing', the strongest correlation is with 'Breathlessness'. Finally it is worth noting that there is a marked clustering of values above and away from the regression line in the two scatter plots, GWB vs. Cough, and GWB vs. Sputum. On inspection of the raw data it was noted that the vast majority of these cases were from Subject 08.

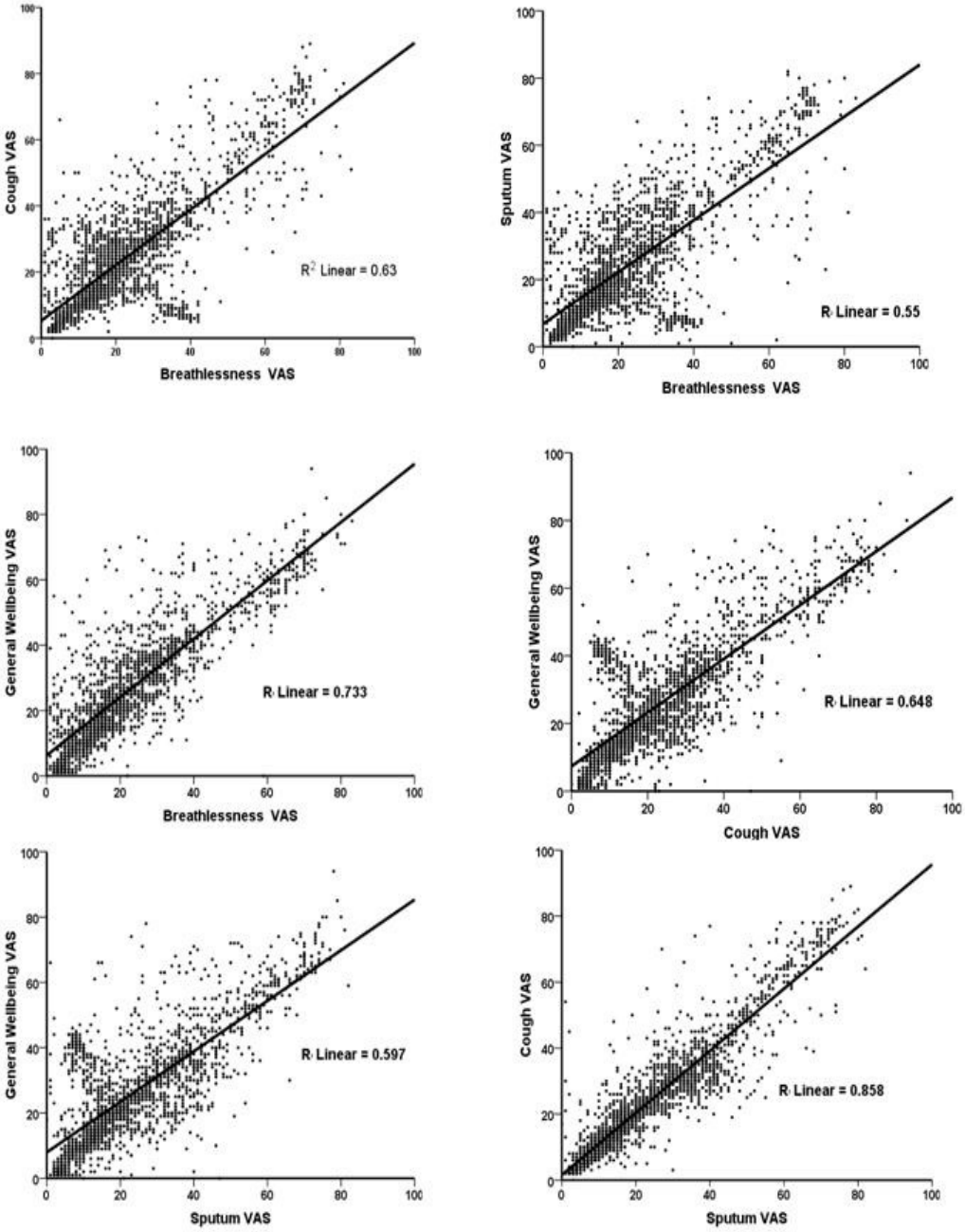

Figure 4-3 Scatter plot correlations for four symptom domains.

4.2 Individual Subjects' Response Profile Plots

A summary Response Profile Plots (RPP) for each subject was plotted, with each point presenting the sum of the four VAS scores for that day. Each of the four VAS scores was plotted for each subject as RPPs, and these individual plots can be found in the Appendix (Section 9.4 below). Spirometric values for each subject for the whole study period are presented. As are described below, there are a number of episodes recorded where symptoms worsened considerably, and then improved without treatment for a CFPE. This may or may not have been associated with a decline in lung function. Such episodes will be henceforth described as pseudo-exacerbations if they last for at least 7 days.

4.2.1 Subject 01

Subject 01 had three CFPE in the study period. Symptoms scores rose gradually over the first 95 days of the study period, and peaked during treatment with IV antibiotics during the first CFPE. A gradual downward decline in lung function was also observed in this period that improved at the start of treatment, but decline again immediately afterwards. Symptoms worsened again shortly after the end of the first CFPE treatment period, but declined again at the start of the second CFPE treatment period. Between the second and third period symptoms worsened, and there appears to have been a pseudo-exacerbation, without decline in lung function, around days 180-200. After this pseudo-exacerbation, symptoms improved again before deteriorating again and treatment for a CFPE was started. Symptoms improved with treatment, but a further pseudo-exacerbation occurred at around days 290-315.

Table 4-3 shows the correlation between individual components of the CMF for subject 01. It can be seen that there is reasonable correlation between the different VAS scores, with rho ranging from 0.552 and 0.809. The sum of VAS scores correlated even better with the other individual components VASs, and also had the best correlation with FEV1 of all the VAS measures, although not the quite the best correlation with FEV6. Interestingly, subject 01 also had moderate correlations between recorded daily temperatures and all of the other components of the CMF. These were in a biologically plausible direction, with increased temperature correlating with increased symptoms and decreased lung function. This correlation was not a universal finding among other subjects.

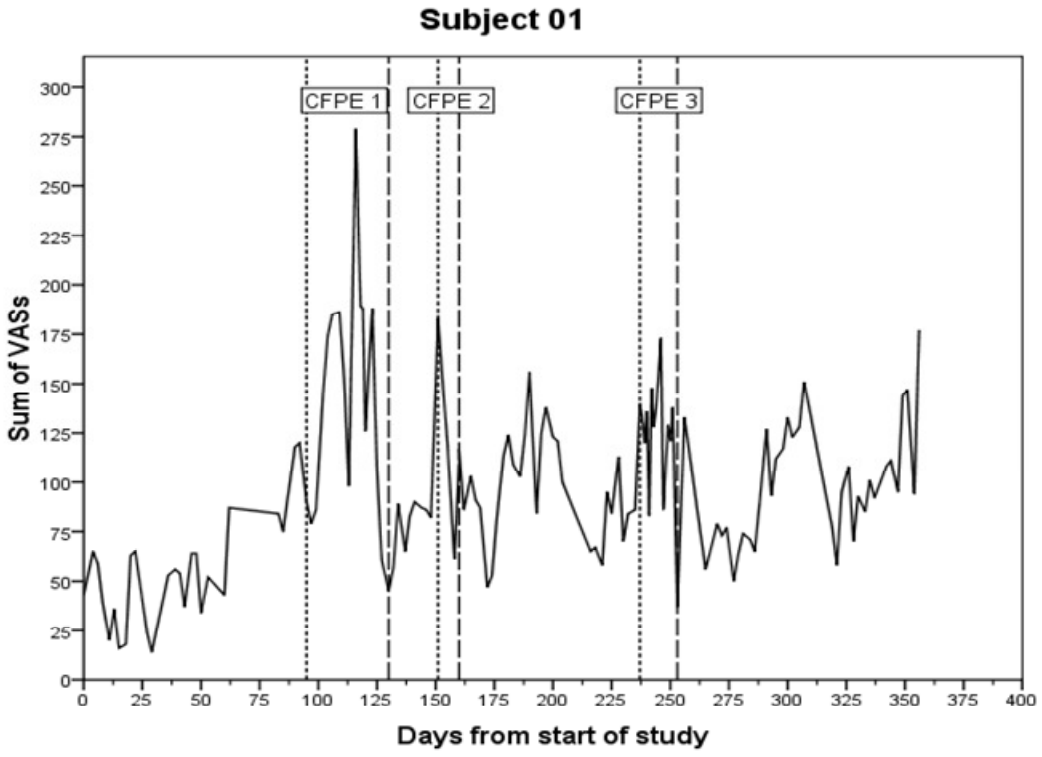

Figure 4-4 Subject 01: Response Profile Plot for Sum of VASs

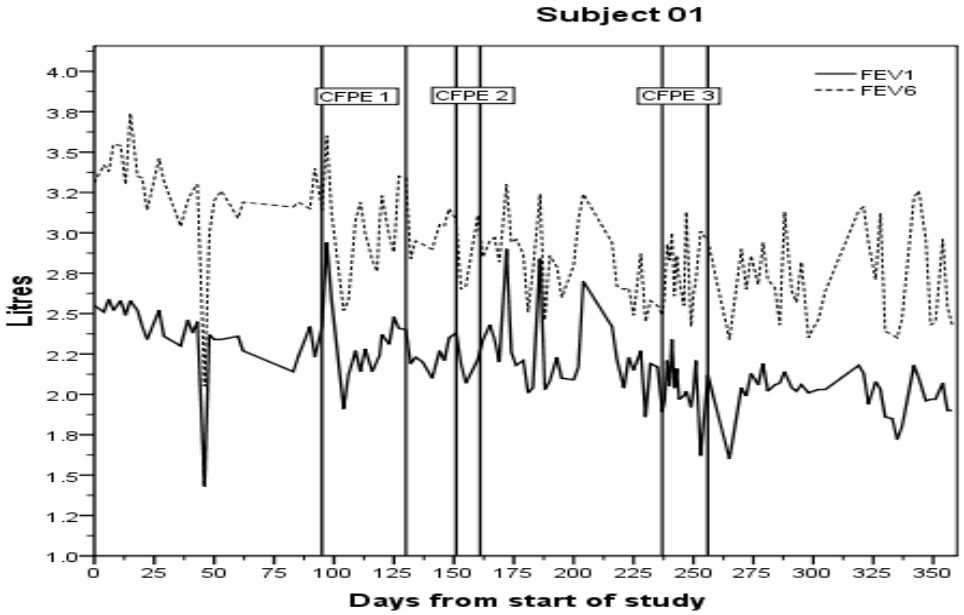

Figure 4-5 Subject 01: Response Profile Plot for FEV1 and FEV6

Subject 01

		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.608****	**.669****	**.552****	**.802****	**-.378****	**-.464****	**.467****
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**
	N.	134	**134**	**134**	**134**	**134**	**130**	**130**	**131**
Cough VAS	rho	.608**	1.000	**.809****	**.619****	**.861****	**-.329****	**-.341****	**.293****
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.001**
	N.	134	134	**134**	**134**	**134**	**130**	**130**	**131**
Sputum VAS	rho	.669**	.809**	1.000	**.607****	**.879****	**-.361****	**-.372****	**.318****
	p value	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**
	N.	134	134	134	**134**	**134**	**130**	**130**	**131**
GWB VAS	rho	.552**	.619**	.607**	1.000	**.844****	**-.331****	**-.390****	**.354****
	p value	<.001	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**
	N.	134	134	134	134	**134**	**130**	**130**	**131**
Sum of VASs	rho	.802**	.861**	.879**	.844**	1.000	**-.397****	**-.448****	**.415****
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**<.001**	**<.001**
	N.	134	134	134	134	134	**130**	**130**	**131**
FEV1	rho	-.378**	-.329**	-.361**	-.331**	-.397**	1.000	**.791****	**-.273****
	p value	<.001	<.001	<.001	<.001	<.001	.	**<.001**	**.002**
	N.	130	130	130	130	130	132	**132**	**129**
FEV6	rho	-.464**	-.341**	-.372**	-.390**	-.448**	.791**	1.000	**-.292****
	p value	<.001	<.001	<.001	<.001	<.001	<.001	.	**.001**
	N.	130	130	130	130	130	132	132	**129**
Temp.	rho	.467**	.293**	.318**	.354**	.415**	-.273**	-.292**	1.000
	p value	<.001	.001	<.001	<.001	<.001	.002	.001	.
	N.	131	131	131	131	131	129	129	133

Table 4-3 Subject 01: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05. Temp. = temperature.

4.2.2 Subject 02

Subject 02 had three courses of antibiotics for CFPEs during the study period. The first coincided with the start of the study. Attempts had been made to recruit subjects who were not having a CFPE. This subject was indeed recruited when CFPE-free, but by the time the booking visit had been arranged a clinical decision had been made to commence treatment for a CFPE.

Symptoms fell from a high level during treatment for the first CFPE, only to peak again at around 80 days, and again fall with treatment. This was associated with a decline in lung function. Symptoms rose gradually between days 150 –

250, prior to treatment for the third CFPE. Again this was associated with marked decline in lung function. Lung function improved with treatment for both the second and third CFPEs. Symptoms remained generally low for most of the remainder of the study period, although two peaks of potential pseudo-exacerbation are noted, one at around days 135 and the other at around day 355. Support for these being classified as pseudo-exacerbations comes from a relative decline in lung function at around the same time.

Table 4-4 shows the correlations between the individual components of the CMF for subject 02. VASs scores show strong correlate win each other, particularly Breathlessness VAS with Cough VAS. The sum of VAS score, however, correlates even more strongly with correlations to three of the four individual VAS scores having a rho of ≥0.9. Sum of VAS also correlated moderately well with spirometry, with a rho value to FEV1 of -0.486, a value marginally higher than for any of the individual VAS scores.

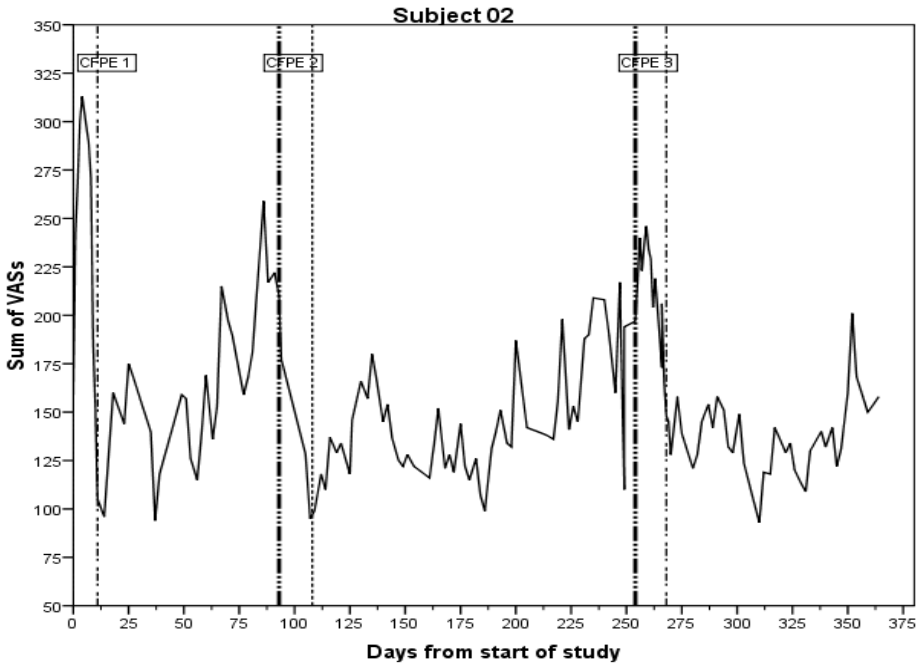

Figure 4-6 Subject 02: Response Profile Plot for Sum of VASs

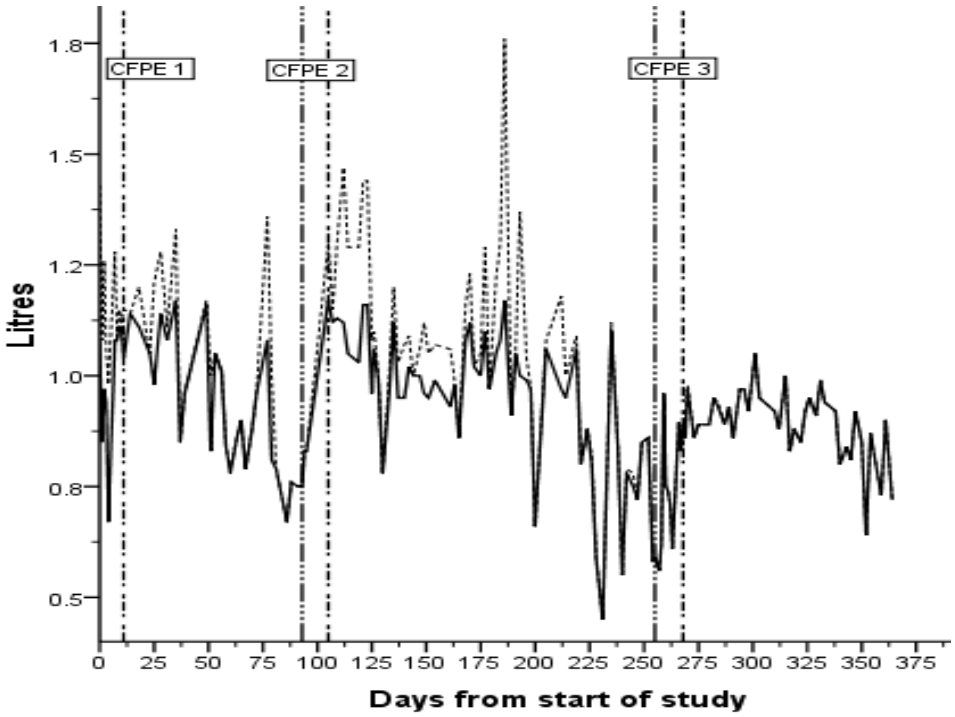

Figure 4-7 Subject 02: Response Profile Plot for FEV1 and FEV6

Subject 02

		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp
Breath VAS	rho	1.000	**.852****	**.362****	**.819****	**.918****	**-.458****	**-.389****	**.210***
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.011**
	N.	148	**148**	**148**	**148**	**148**	**146**	**145**	**147**
Cough VAS	rho	.852**	1.000	**.406****	**.795****	**.900****	**-.412****	**-.337****	**.204***
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.013**
	N.	148	148	**148**	**148**	**148**	**146**	**145**	**147**
Sputum VAS	rho	.362**	.406**	1.000	**.424****	**.573****	**-.254****	**-.234****	**.035**
	p value	<.001	<.001	.	**<.001**	**<.001**	**.002**	**.005**	**.672**
	N.	148	148	148	**148**	**148**	**146**	**145**	**147**
GWB VAS	rho	.819**	.795**	.424**	1.000	**.925****	**-.484****	**-.433****	**.237****
	p value	<.001	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**.004**
	N.	148	148	148	148	**148**	**146**	**145**	**147**
Sum of VASs	rho	.918**	.900**	.573**	.925**	1.000	**-.486****	**-.418****	**.217****
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**<.001**	**.008**
	N.	148	148	148	148	148	**146**	**145**	**147**
FEV1	rho	-.458**	-.412**	-.254**	-.484**	-.486**	1.000	**.936****	**-.109**
	p value	<.001	<.001	.002	<.001	<.001	.	**<.001**	**.187**
	N.	146	146	146	146	146	149	**148**	**148**
FEV6	rho	-.389**	-.337**	-.234**	-.433**	-.418**	.936**	1.000	**-.100**
	p value	<.001	<.001	.005	<.001	<.001	<.001	.	**.226**
	N.	145	145	145	145	145	148	148	**147**
Temp.	rho	.210*	.204*	.035	.237**	.217**	-.109	-.100	1.000
	p value	.011	.013	.672	.004	.008	.187	.226	.
	N.	147	147	147	147	147	148	147	150

Table 4-4 Subject 02: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05

4.2.3 Subject 03

Subject 03 had no CFPEs treated in the study period. However, there is clearly a large worsening in the symptoms between days 210 – 265, consistent with a pseudo-exacerbation. This is not associated with decline in lung function, and improves spontaneously.

Figure Table 4-5 below shows the correlations between the various components of the CMF. Of note, the Sum of VAS score performed marginally better in

correlations with other individual VAS components and FEV1, than any individual components.

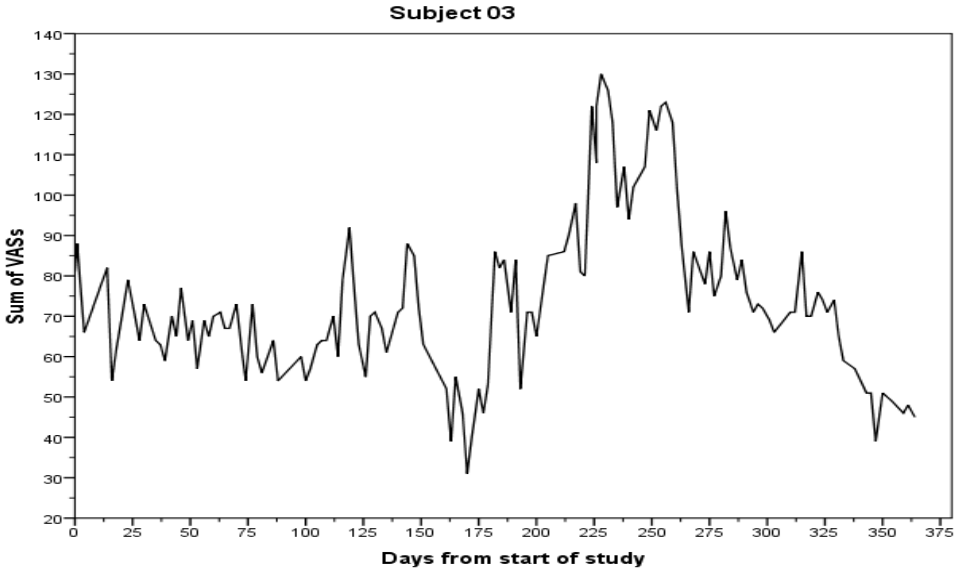

Figure 4-8 Subject 03: Response Profile Plot for Sum of VASs

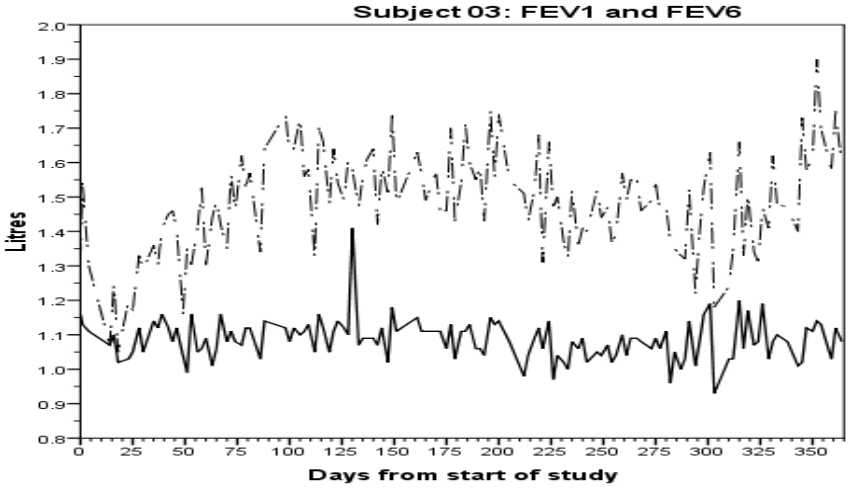

Figure 4-9 Subject 03: Response Profile Plot for FEV1 (solid line) and FEV6 (dashed line)

Subject 03		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp
Breath VAS	rho	1.000	**.843****	**.594****	**.779****	**.884****	**-.245****	**-.215***	**.212***
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**.004**	**.012**	**.014**
	N.	135	**135**	**135**	**135**	**135**	**135**	**135**	**135**
Cough VAS	rho	.843**	1.000	**.713****	**.786****	**.947****	**-.295****	**-.193***	**.197***
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**.001**	**.025**	**.022**
	N.	135	135	**135**	**135**	**135**	**135**	**135**	**135**
Sputum VAS	rho	.594**	.713**	1.000	**.643****	**.805****	**-.260****	**-.125**	**.218***
	p value	<.001	<.001	.	**<.001**	**<.001**	**.002**	**.147**	**.011**
	N.	135	135	135	**135**	**135**	**135**	**135**	**135**
GWB VAS	rho	.779**	.786**	.643**	1.000	**.874****	**-.273****	**-.202***	**.302****
	p value	<.001	<.001	<.001	.	**<.001**	**.001**	**.019**	**<.001**
	N.	135	135	135	135	**135**	**135**	**135**	**135**
Sum of VASs	rho	.884**	.947**	.805**	.874**	1.000	**-.298****	**-.210***	**.248****
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**.015**	**.004**
	N.	135	135	135	135	135	**135**	**135**	**135**
FEV1	rho	-.245**	-.295**	-.260**	-.273**	-.298**	1.000	**.519****	**-.171***
	p value	.004	.001	.002	.001	<.001	.	**<.001**	**.047**
	N.	135	135	135	135	135	135	**135**	**135**
FEV6	rho	-.215*	-.193*	-.125	-.202*	-.210*	.519**	1.000	**.098**
	p value	.012	.025	.147	.019	.015	<.001	.	**.256**
	N.	135	135	135	135	135	135	135	**135**
Temp.	rho	.212*	.197*	.218*	.302**	.248**	-.171*	.098	1.000
	p value	.014	.022	.011	<.001	.004	.047	.256	.
	N.	135	135	135	135	135	135	135	135

Table 4-5 Subject 03: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05

4.2.4 Subject 04

As has been noted previously, Subject 04 was not as productive of sputum (and therefore CMFs) as had been hoped. However, useful data was obtained none the less. There were three treated CFPEs, all of which were associated with worsening of symptoms. The first two can be seen to improve with treatment, although briefly for the first. The third CFPE coincided with the end of the study period, and data for the post-CFPE period was not obtained. There was little in the way of change in lung function associated with either the diagnosis of a CFPE, or in response to treatment. The data was too patchy to be sure of the presence of any pseudo-exacerbations. Table 4-6 below show the correlations

between the various components of the CMF for subject 04. This confirms the impression from visual inspection of the RPPs, that there is little or no correlation between Sum of VAS scores, or any individual VAS scores, and spirometry. There are a number of expected strong correlations between individual VASs, but no correlation between any of these and any objective measure.

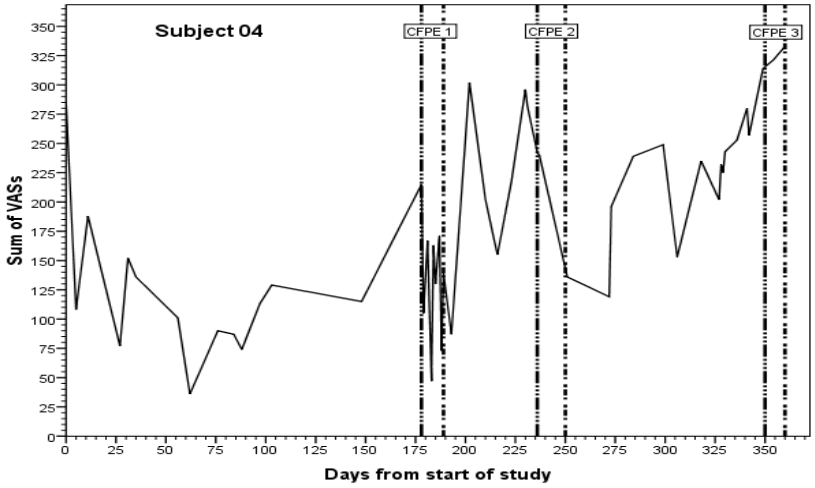

Figure 4-10 Subject 04: Response Profile Plot for Sum of VASs

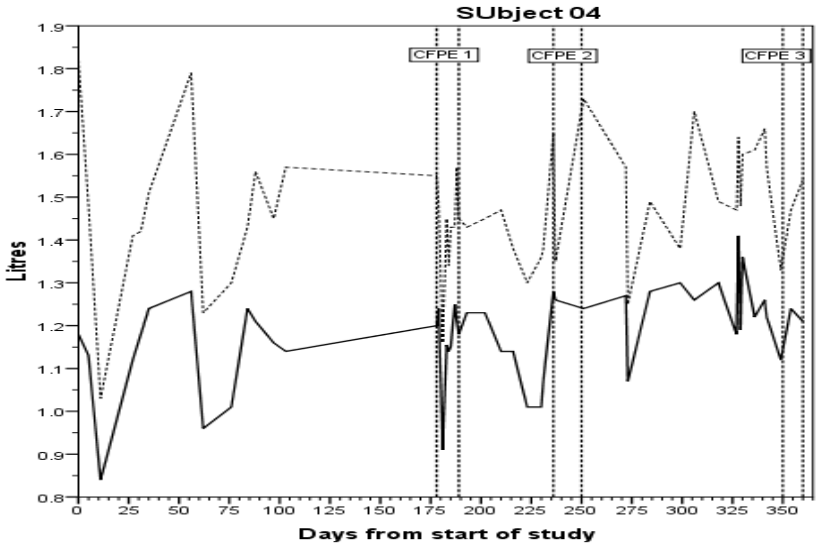

Figure 4-11 Subject 04: Response Profile Plot for FEV1 (solid line) and FEV6 (dashed line)

Subject 04

		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.681****	**.533****	**.714****	**.802****	**.190**	**.027**	**-.182**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**.181**	**.850**	**.197**
	N.	52	**52**	**52**	**52**	**52**	**51**	**51**	**52**
Cough VAS	rho	.681**	1.000	**.804****	**.757****	**.916****	**.274**	**.082**	**-.006**
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**.052**	**.566**	**.967**
	N.	52	52	**52**	**52**	**52**	**51**	**51**	**52**
Sputum VAS	rho	.533**	.804**	1.000	**.797****	**.890****	**.117**	**.073**	**.191**
	p value	<.001	<.001	.	**<.001**	**<.001**	**.413**	**.610**	**.176**
	N.	52	52	52	**52**	**52**	**51**	**51**	**52**
GWB VAS	rho	.714**	.757**	.797**	1.000	**.917****	**.106**	**-.014**	**-.037**
	p value	<.001	<.001	<.001	.	**<.001**	**.461**	**.924**	**.793**
	N.	52	52	52	52	**52**	**51**	**51**	**52**
Sum of VASs	rho	.802**	.916**	.890**	.917**	1.000	**.204**	**.053**	**-.014**
	p value	<.001	<.001	<.001	<.001	.	**.151**	**.714**	**.922**
	N.	52	52	52	52	52	**51**	**51**	**52**
FEV1	rho	.190	.274	.117	.106	.204	1.000	**.623****	**-.183**
	p value	.181	.052	.413	.461	.151	.	**<.001**	**.198**
	N.	51	51	51	51	51	51	**51**	**51**
FEV6	rho	.027	.082	.073	-.014	.053	.623**	1.000	**-.090**
	p value	.850	.566	.610	.924	.714	<.001	.	**.532**
	N.	51	51	51	51	51	51	51	**51**
Temp.	rho	-.182	-.006	.191	-.037	-.014	-.183	-.090	1.000
	p value	.197	.967	.176	.793	.922	.198	.532	.
	N.	52	52	52	52	52	51	51	52

Table 4-6 Subject 04: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.5 Subject 05

Subject 05 had five treated CFPEs during the study period, with exacerbations 1, 4, and 5 being treated either with IV antibiotics alone or oral and then IV antibiotics. CFPEs 1, 4 and 5 were also the episodes that were associated with the largest deteriorations in symptoms at initiation of treatment, and response to therapy. Of note, for all three of the IV treated CFPEs, the symptom scores became worse following the initiation of treatment before an improvement back to baseline. In addition, all three IV treated CFPEs were associated with decline in lung function, with even lower values during treatment that gradually,

if only partially recovered. The first CFPE, which was associated with the worst symptoms and the lowest lung function, lasted 42 days and required 3 consecutive different antibiotic combinations.

Correlations between the individual components of the CMF for subject 05 are presented in Table 4-7 below. This confirms the visual inspection of the RPPs, with strong negative correlations between the Sum of VAS and both FEV1 and FEV6 (rho -0.484 and -0.545 respectively). Unlike some other subjects, however, there was no correlation between temperature and symptoms or lung function.

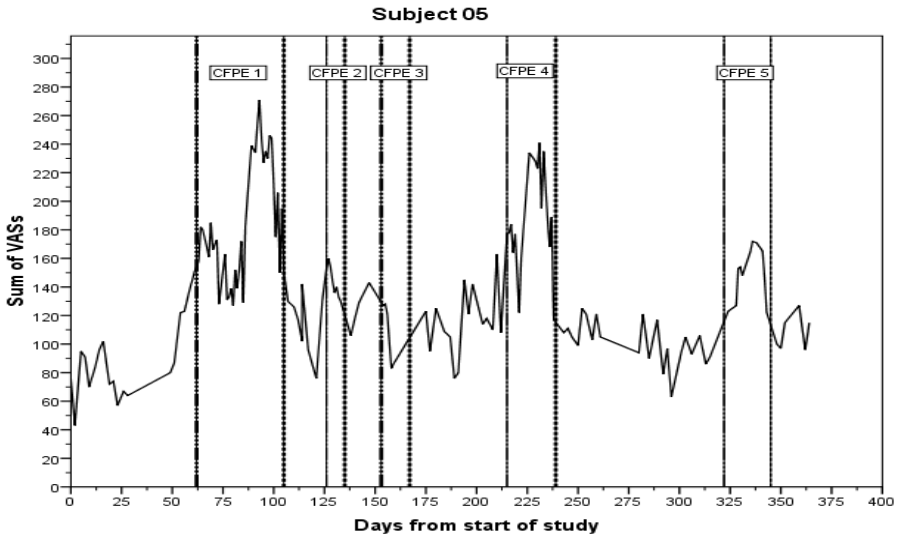

Figure 4-12 Subject 05: Response Profile Plot for Sum of VASs

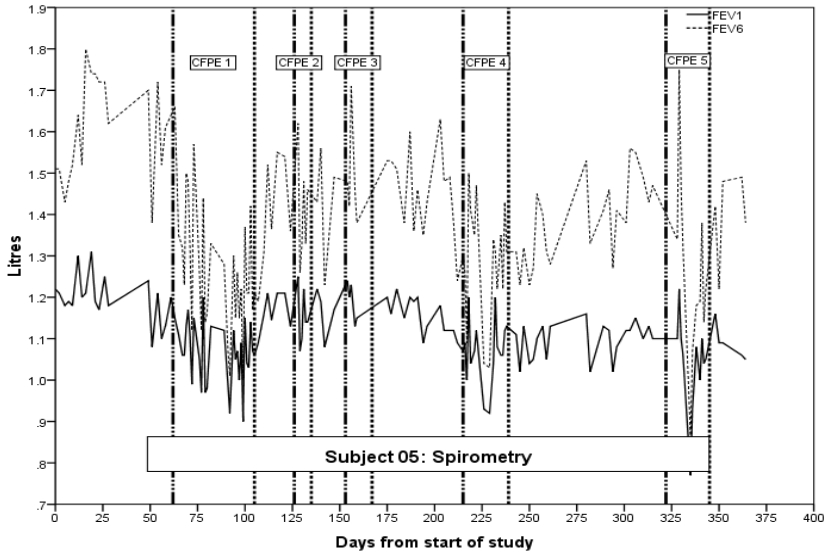

Figure 4-13 Subject 05: Response Profile Plot for FEV1 and FEV6

Subject 05		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.757****	**.725****	**.651****	**.848****	**-.499****	**-.539****	**.098**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.234**
	N.	153	**153**	**153**	**152**	**152**	**140**	**140**	**150**
Cough VAS	rho	.757**	1.000	**.840****	**.706****	**.908****	**-.431****	**-.494****	**.106**
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.198**
	N.	153	153	**153**	**152**	**152**	**140**	**140**	**150**
Sputum VAS	rho	.725**	.840**	1.000	**.694****	**.892****	**-.418****	**-.491****	**.030**
	p value	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**.718**
	N.	153	153	153	**152**	**152**	**140**	**140**	**150**
GWB VAS	rho	.651**	.706**	.694**	1.000	**.890****	**-.442****	**-.461****	**.179***
	p value	<.001	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**.029**
	N.	152	152	152	152	**152**	**139**	**139**	**149**
Sum of VASs	rho	.848**	.908**	.892**	.890**	1.000	**-.484****	**-.545****	**.136**
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**<.001**	**.098**
	N.	152	152	152	152	152	**139**	**139**	**149**
FEV1	rho	-.499**	-.431**	-.418**	-.442**	-.484**	1.000	**.816****	**.037**
	p value	<.001	<.001	<.001	<.001	<.001	.	**<.001**	**.667**
	N.	140	140	140	139	139	140	**140**	**137**
FEV6	rho	-.539**	-.494**	-.491**	-.461**	-.545**	.816**	1.000	**-.004**
	p value	<.001	<.001	<.001	<.001	<.001	<.001	.	**.964**
	N.	140	140	140	139	139	140	140	**137**
Temp.	rho	.098	.106	.030	.179*	.136	.037	-.004	1.000
	p value	.234	.198	.718	.029	.098	.667	.964	.
	N.	150	150	150	149	149	137	137	150

Table 4-7 Subject 05: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.6 Subject 06

Subject 06 had two treated CFPEs, both treated with oral antibiotics. There appears to have been a large deterioration in symptoms between days 75 – 100 which is only associated with a small gradual decline in lung function. However, the first treated CFPE occurs at day 210, the second at day 255. The first course of treatment is associated with a short lived improvement in symptoms, whilst the second course of treatment is associated with a longer lasting improvement. There is a further deterioration in symptoms at around day 340

that improves without treatment and is not associated with a measurable decline in lung function. This is consistent with a pseudo-exacerbation.

Correlations between the components of the CMF for subject 06 are presented in Table 4-8 below. This shows very strong correlations between the individual VAS scores, and moderate correlations with lung function. However, although Sum of VAS does correlate relatively well with FEV1 and FEV6 (rho -0.392 and -0.390 respectively), both Breathlessness VAS and General Wellbeing VASs correlate more strongly (rho up to -0.478).

It can be noted that there is a long delay from a considerable increase in symptoms consistent with a CFPE, and instigation of treatment (oral antibiotics). Discussion with the clinical team revealed that this CFPE was diagnosed and treated at a routine out patients' appointment visit. This subject only reported the increase in symptoms of direct questioning by the clinician. It was not self-recognised as a CFPE.

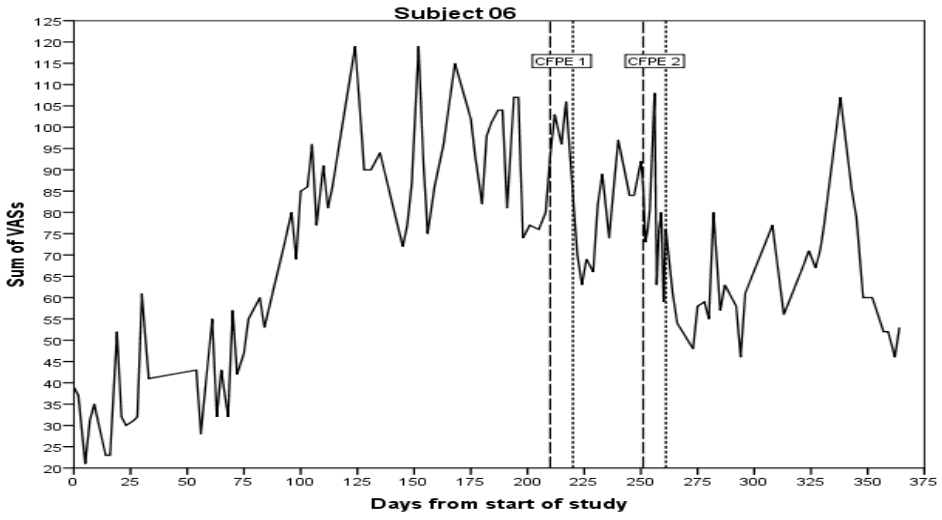

Figure 4-14 Subject 06: Response Profile Plot for Sum of VASs

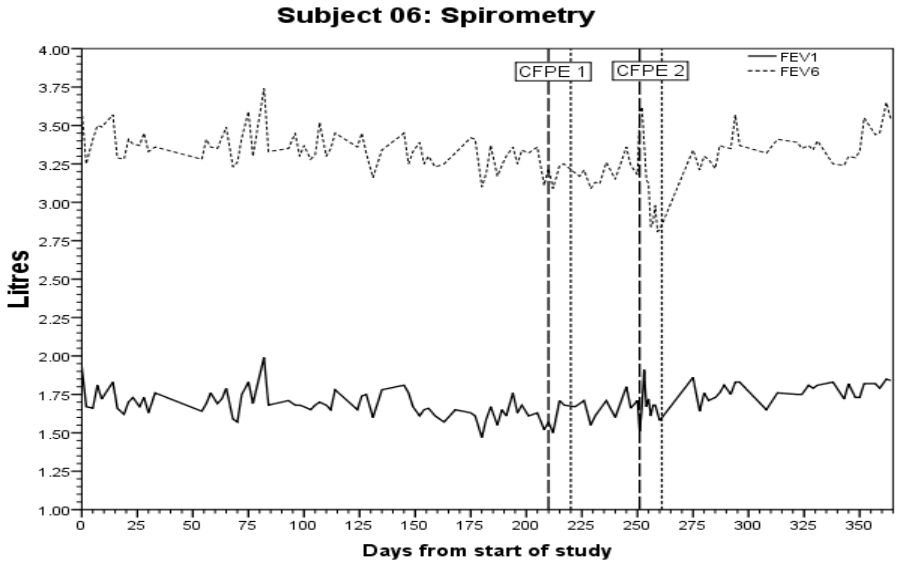

Figure 4-15 Subject 06: Response Profile Plot for FEV1 and FEV6

Subject 06

		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp
Breath VAS	rho	1.000	**.904****	**.735****	**.820****	**.927****	**-.410****	**-.387****	**-.048**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.602**
	N.	121	**121**	**121**	**121**	**121**	**117**	**117**	**121**
Cough VAS	rho	.904**	1.000	**.831****	**.817****	**.947****	**-.367****	**-.371****	**-.104**
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.258**
	N.	121	121	**121**	**121**	**121**	**117**	**117**	**121**
Sputum VAS	rho	.735**	.831**	1.000	**.741****	**.854****	**-.208***	**-.298****	**-.049**
	p value	<.001	<.001	.	**<.001**	**<.001**	**.025**	**.001**	**.591**
	N.	121	121	121	**121**	**121**	**117**	**117**	**121**
GWB VAS	rho	.820**	.817**	.741**	1.000	**.874****	**-.415****	**-.478****	**-.085**
	p value	<.001	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**.356**
	N.	121	121	121	121	**121**	**117**	**117**	**121**
Sum of VASs	rho	.927**	.947**	.854**	.874**	1.000	**-.392****	**-.390****	**-.069**
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**<.001**	**.451**
	N.	121	121	121	121	121	**117**	**117**	**121**
FEV1	rho	-.410**	-.367**	-.208*	-.415**	-.392**	1.000	**.578****	**.049**
	p value	<.001	<.001	.025	<.001	<.001	.	**<.001**	**.599**
	N.	117	117	117	117	117	117	**117**	**117**
FEV6	rho	-.387**	-.371**	-.298**	-.478**	-.390**	.578**	1.000	**.131**
	p value	<.001	<.001	.001	<.001	<.001	<.001	.	**.160**
	N.	117	117	117	117	117	117	117	**117**
Temp.	rho	-.048	-.104	-.049	-.085	-.069	.049	.131	1.000
	p value	.602	.258	.591	.356	.451	.599	.160	.
	N.	121	121	121	121	121	117	117	121

Table 4-8 Subject 06: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.7 Subject 07

Subject 07 had four treated CFPEs. The first two were treated with IV antibiotics and the second two with oral antibiotics. Judged by changes in symptoms and lung function, the second exacerbation was a major health event, with a rise from around 80 to a peak of 280 for the sum of the VAS scores, and a fall in FEV1 from around 1.8L to a nadir of less than 1.4L.. Symptoms improved more quickly after this second CFPE than lung function,

improving within a few days compared to 30 or more days for a recovery in FEV1.

Symptom scores responded to all the course of antibiotics, to a greater or lesser degree. There was only one episode which could be described as a pseudo-exacerbation: around 14 days after the end of the second CFPE there was a large deterioration in symptom scores, greater in magnitude than all but the second CFPE. This resolved within 10 days of starting. There was no change in lung function with this pseudo-exacerbation.

Table 4-9 shows the correlations between the various components of the CMF. Compared to some other subjects 09's VAS scores do not correlate as strongly with each other (rho 0.478 to 0.844). However, particularly noteworthy is the very strong negative correlation between Breathlessness VAS and FEV1 (rho -0.720). It is also worth noting that there are some weak positive correlations between temperature and Cough and Sputum VAS scores (rho 0.217 and 0.232 respectively), which seem biologically plausible. However there is also a statistically significant weak (rho 0.184) positive correlation between temperature and FEV1 (but not FEV6) which is less biologically plausible.

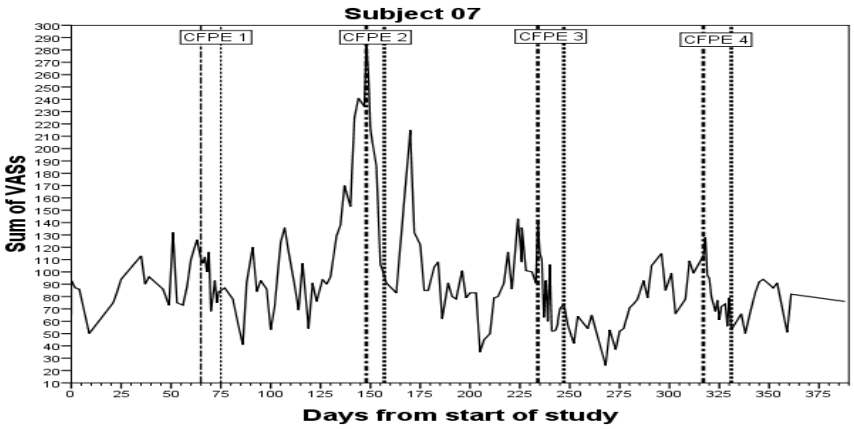

Figure 4-16 Subject 07: Response Profile Plot for Sum of VASs

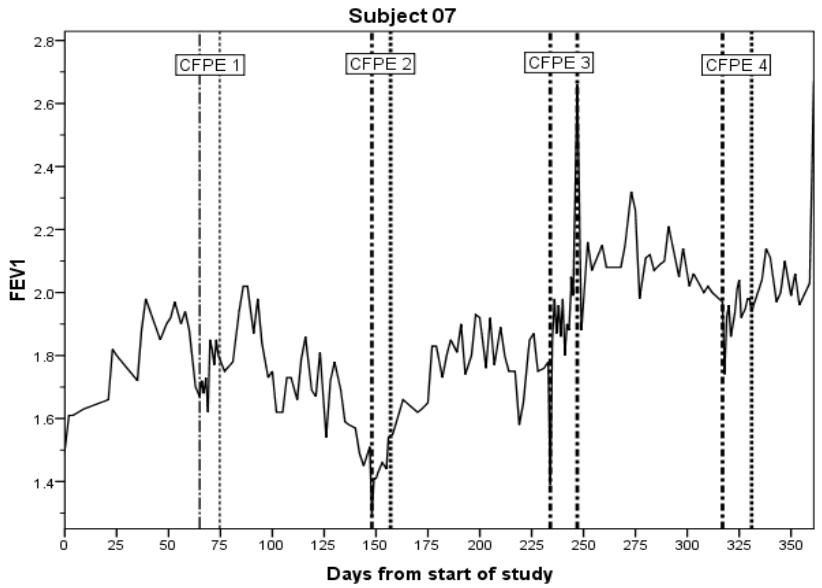

Figure 4-17 Subject 07: Response Profile Plot for FEV1 and FEV6

Subject 07		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.478****	**.333****	**.553****	**.680****	**-.720****	**-.641****	**-.119**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.137**
	N.	159	**159**	**159**	**159**	**159**	**159**	**159**	**159**
Cough VAS	rho	.478**	1.000	**.844****	**.674****	**.889****	**-.335****	**-.298****	**.217****
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.006**
	N.	159	159	**159**	**159**	**159**	**159**	**159**	**159**
Sputum VAS	rho	.333**	.844**	1.000	**.611****	**.829****	**-.244****	**-.218****	**.232****
	p value	<.001	<.001	.	**<.001**	**<.001**	**.002**	**.006**	**.003**
	N.	159	159	159	**159**	**159**	**159**	**159**	**159**
GWB VAS	rho	.553**	.674**	.611**	1.000	**.856****	**-.487****	**-.493****	**.167***
	p value	<.001	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**.035**
	N.	159	159	159	159	**159**	**159**	**159**	**159**
Sum of VASs	rho	.680**	.889**	.829**	.856**	1.000	**-.522****	**-.482****	**.146**
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**<.001**	**.067**
	N.	159	159	159	159	159	**159**	**159**	**159**
FEV1	rho	-.720**	-.335**	-.244**	-.487**	-.522**	1.000	**.930****	**.184***
	p value	<.001	<.001	.002	<.001	<.001	.	**<.001**	**.020**
	N.	159	159	159	159	159	159	**159**	**159**
FEV6	rho	-.641**	-.298**	-.218**	-.493**	-.482**	.930**	1.000	**.181***
	p value	<.001	<.001	.006	<.001	<.001	<.001	.	**.022**
	N.	159	159	159	159	159	159	159	**159**
Temp.	rho	-.119	.217**	.232**	.167*	.146	.184*	.181*	1.000
	p value	.137	.006	.003	.035	.067	.020	.022	.
	N.	159	159	159	159	159	159	159	159

Table 4-9 Subject 07: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.8 Subject 08

Subject 08 had no treated CFPEs during the study period, which extended to 450 days. He received one course of oral antibiotics for an unrelated presumed staphylococcal skin infection. The Sum of Symptoms and FEV1 RPPs were very consistent (compared to other subjects) through the study period. A more in depth look at individual VAS plots shoed an upward trend for Breathlessness and General Wellbeing, but downward trend for cough and sputum. These divergent trends appeared to cancel each other out in the Sum of VAS plot. There appeared to be a general trend of increasing FEV6 values though out the year, but stable FEV1 values. There did not appear to be any pseudo-exacerbations.

The visual inspection of the individual RPPs for subject 08 suggest different factors at play, as noted above. These perceptions are strengthened by the results of the correlations between the components of the CMF, presented in Table 4-10. From this it can be seen that there is a biologically plausible negative correlation between Cough and Sputum VAS scores with FEV1 (rho -0.434 -0.489 respectively) and biologically implausible positive correlations between Breathlessness and General Wellbeing scores with FEV1 (rho 0.24 and 0.214 respectively). It is worth noting, that for this subject, FEV6 correlated more strongly with all other measures than FEV1.

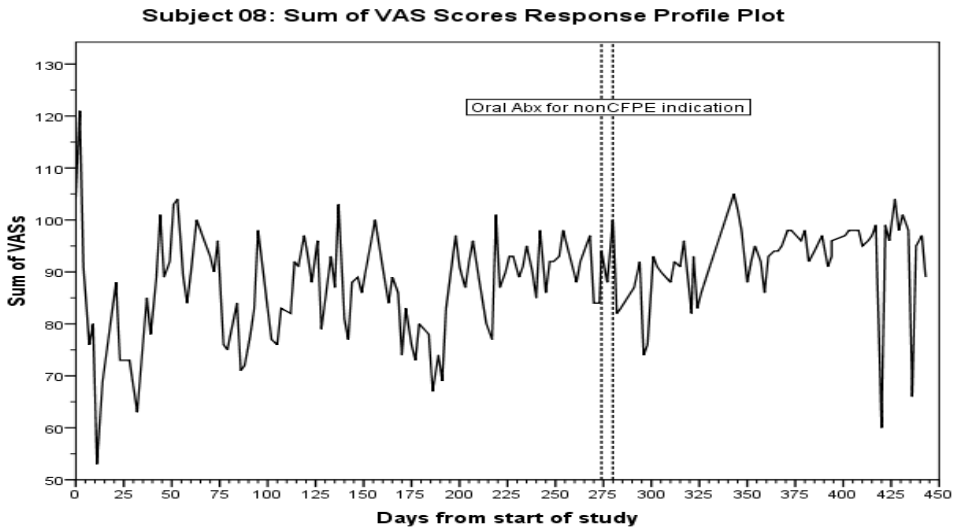

Figure 4-18 Subject 08: Response Profile Plot for Sum of VASs

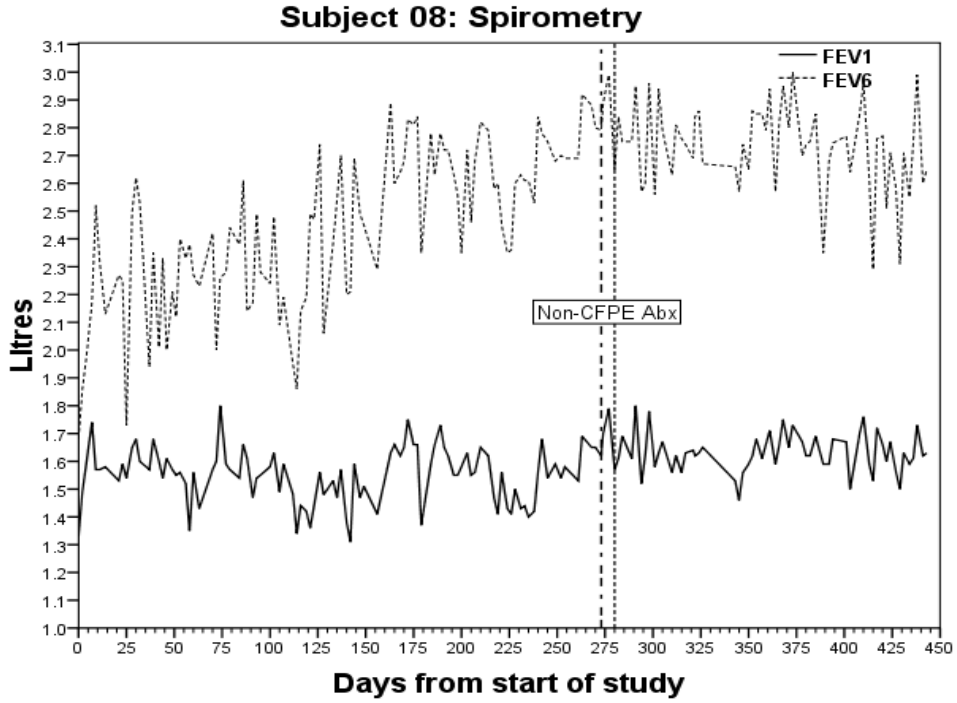

Figure 4-19 Subject 08: Response Profile Plot for FEV1 and FEV6.

Subject 08

		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**-.689****	**-.563****	**.826****	**.659****	**.240****	**.506****	**-.040**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**.002**	**<.001**	**.623**
	N.	159	**159**	**159**	**159**	**159**	**158**	**158**	**156**
Cough VAS	rho	-.689**	1.000	**.823****	**-.689****	**-.097**	**-.434****	**-.634****	**.083**
	p value	<.001	.	**<.001**	**<.001**	**.224**	**<.001**	**<.001**	**.303**
	N.	159	159	**159**	**159**	**159**	**158**	**158**	**156**
Sputum VAS	rho	-.563**	.823**	1.000	**-.534****	**.057**	**-.489****	**-.628****	**.096**
	p value	<.001	<.001	.	**<.001**	**.478**	**<.001**	**<.001**	**.234**
	N.	159	159	159	**159**	**159**	**158**	**158**	**156**
GWB VAS	rho	.826**	-.689**	-.534**	1.000	**.651****	**.214****	**.523****	**-.056**
	p value	<.001	<.001	<.001	.	**<.001**	**.007**	**<.001**	**.491**
	N.	159	159	159	159	**159**	**158**	**158**	**156**
Sum of VASs	rho	.659**	-.097	.057	.651**	1.000	**-.106**	**.084**	**.076**
	p value	<.001	.224	.478	<.001	.	**.184**	**.293**	**.348**
	N.	159	159	159	159	159	**158**	**158**	**156**
FEV1	rho	.240**	-.434**	-.489**	.214**	-.106	1.000	**.648****	**.004**
	p value	.002	<.001	<.001	.007	.184	.	**<.001**	**.961**
	N.	158	158	158	158	158	160	**159**	**157**
FEV6	rho	.506**	-.634**	-.628**	.523**	.084	.648**	1.000	**-.113**
	p value	<.001	<.001	<.001	<.001	.293	<.001	.	**.159**
	N.	158	158	158	158	158	159	160	**157**
Temp.	rho	-.040	.083	.096	-.056	.076	.004	-.113	1.000
	p value	.623	.303	.234	.491	.348	.961	.159	.
	N.	156	156	156	156	156	157	157	158

Table 4-10 Subject 08: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.9 Subject 09

Subject 09 had some difficulties with completing the Clinical Monitoring Form. For the first 220 days of the study he scored almost zero across all four VAS domains, yet had a number of IV antibiotic courses for CFPEs, and had the lowest FEV1% predicted. The principle researcher was not keen to apply undue influence on the way subjects complete their subjective symptom scoring. However, it became apparent that the data being recorded was in no way reflecting clinical patterns. The decision was made to discuss Subject 09's

understanding of the process of completing VASs. During this discussion it became clear that he was completing the VAS for how he felt at the moment of filling it in (*i.e.* while sitting), as opposed to how he felt during that day so far. Such a misunderstanding may have be made more likely by his previous record of poor educational attainment and low levels of adherence with appointments and the CF service. The misunderstanding was corrected. VAS data prior to the corrective discussion was regrettably discarded.

In addition to the above difficulties with the VAS process, Subject 09 also had difficulties with the home spirometer. This was problem developed during the course of the study and was not apparent at the start. With his FEV1% predicted being less than 20% at the start of the study, completion of FEV1 and FEV6 caused severely disabling dyspnoea and curtailed his ability and willingness to go through this each morning. Thus by the end of the study he was no longer recording his spirometry.

With regard to the recorded data presented below in Table 4-11, he had 5 separate treated CFPEs. All of these were treated at least in part with IV antibiotics. In addition, he received one course of IV antibiotics that were arranged electively. For his symptoms, there was a clear improvement in symptoms during and after the CFPE treatment that started on day 256. Although there is no clear pattern of an increase leading up to this treated CFPE, there may have been an insufficient period of time with captured data to allow visualisation of any deterioration that preceded the start of treatment. Following the end of treatment at day 279, symptoms began to deteriorate culminating in another treated CFPE on day 336. Symptoms improved considerably with treatment for this CFPE at day 336, and continued to improve

after the end of IV antibiotics at day 357. Symptoms then worsened again at around day 380 before starting to improve around day 390. It was around this time that elective IV antibiotics were started, the decision having been made at or shortly after the end of the previous course of IV antibiotics. During and after the elective course of IV antibiotics, symptoms improved to their lowest level.

Other than the deterioration and subsequent improvement in symptoms that coincided with the elective course of antibiotics there were no apparent episodes of pseudo-exacerbations in the study period. Unfortunately there was no overlap between the available data for symptoms and that available for lung function.

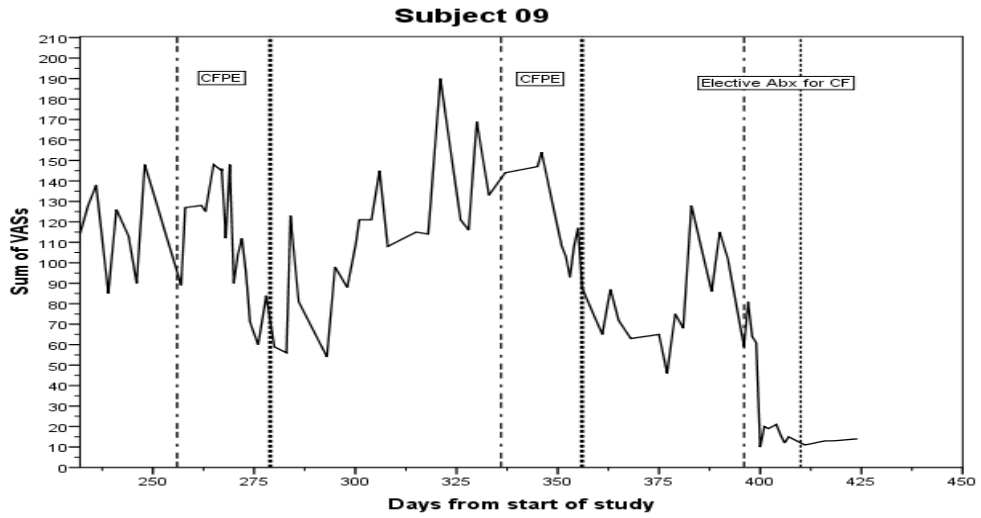

Figure 4-20 Subject 09: Response Profile Plot for Sum of VASs.

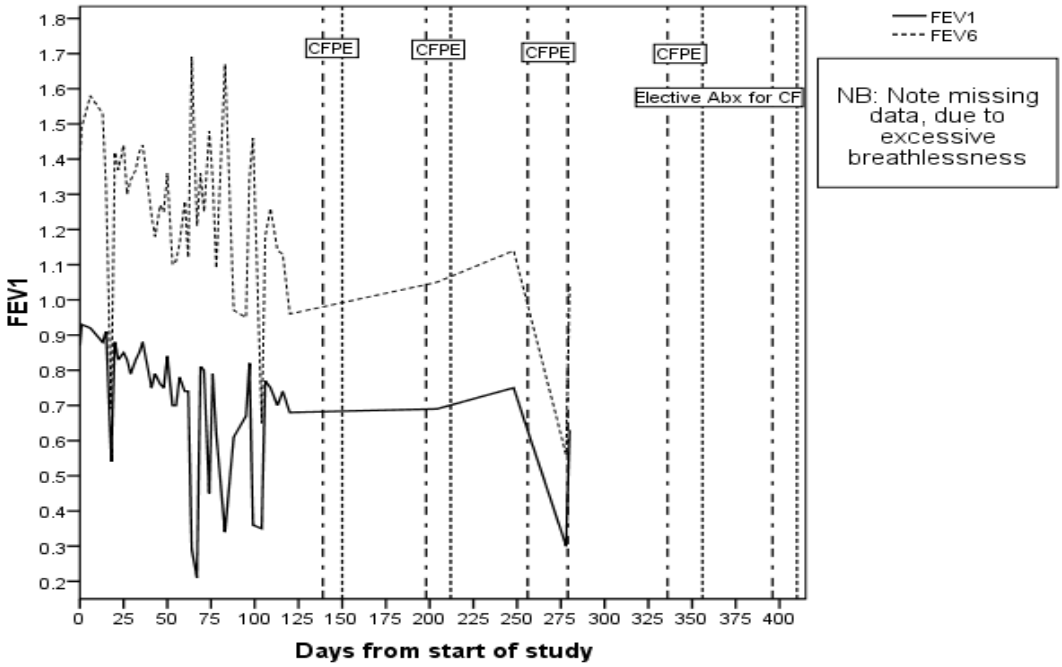

Figure 4-21 Subject 09: Response Profile Plot for FEV1 and FEV6

Subject 09

		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.884****	**.777****	**.736****	**.907****	**.500**	**.500**	**-.148**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**.667**	**.667**	**.222**
	N.	83	**83**	**83**	**83**	**83**	**3**	**3**	**70**
Cough VAS	rho	.884**	1.000	**.839****	**.778****	**.945****	**.500**	**.500**	**-.111**
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**.667**	**.667**	**.359**
	N.	83	83	**83**	**83**	**83**	**3**	**3**	**70**
Sputum VAS	rho	.777**	.839**	1.000	**.761****	**.924****	**1.000****	**1.000****	**-.093**
	p value	<.001	<.001	.	**<.001**	**<.001**	**.**	**.**	**.442**
	N.	83	83	83	**83**	**83**	**3**	**3**	**70**
GWB VAS	rho	.736**	.778**	.761**	1.000	**.876****	**.500**	**.500**	**-.218**
	p value	<.001	<.001	<.001	.	**<.001**	**.667**	**.667**	**.070**
	N.	83	83	83	83	**83**	**3**	**3**	**70**
Sum of VASs	rho	.907**	.945**	.924**	.876**	1.000	**.500**	**.500**	**-.176**
	p value	<.001	<.001	<.001	<.001	.	**.667**	**.667**	**.144**
	N.	83	83	83	83	83	**3**	**3**	**70**
FEV1	rho	.500	.500	1.000**	.500	.500	1.000	**.536****	**.086**
	p value	.667	.667	.	.667	.667	.	**<.001**	**.563**
	N.	3	3	3	3	3	47	**47**	**47**
FEV6	rho	.500	.500	1.000**	.500	.500	.536**	1.000	**-.043**
	p value	.667	.667	.	.667	.667	<.001	.	**.774**
	N.	3	3	3	3	3	47	48	**48**
Temp.	rho	-.148	-.111	-.093	-.218	-.176	.086	-.043	1.000
	p value	.222	.359	.442	.070	.144	.563	.774	.
	N.	70	70	70	70	70	47	48	177

Table 4-11 Subject 09: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.10 Subject 10

Subject 10 had two courses of antibiotics for CFPEs, the first orally and the second intravenously. In addition one course was given intravenously as an elective treatment. For Sum of Symptoms, there was a marked worsening in symptoms at around the time of the start of the course of elective IV antibiotics that was not associated with any perceptible deterioration in spirometry. This deterioration in symptoms was not improved by the elective course of antibiotics. In addition, there were two further courses of antibiotics for CFPEs: there was a further worsening in symptom scores at the time of the first, and a slight improvement at the time of the second. Overall there was little apparent

correlation between values from the CMF and antibiotic treatment, either elective or for CFPEs.

This lack of correlation between CMF values and objective markers such CFPEs lung function and temperature is born out by the correlations between the values presented in Table 4-12 below. There only the weakest of significant positive correlations of VAS scores with FEV1, which is biologically implausible, and a marginally stronger (negative) correlation with FEV6 and therefore more biologically plausible. Despite this lack of correlations between objective and subjective measures, there is very strong correlation between all the VAS scores. One possible explanation for this discrepancy would be that little or no thought was given by subject 10 to the completion of the VASs, and lines were placed in approximately the same for all VASs to make the process as quick as possible. Although a plausible explanation, this would not fit with the experience of those who interacted with the subject, who was considered highly adherent to therapies and related activities, with good educational attainment levels.

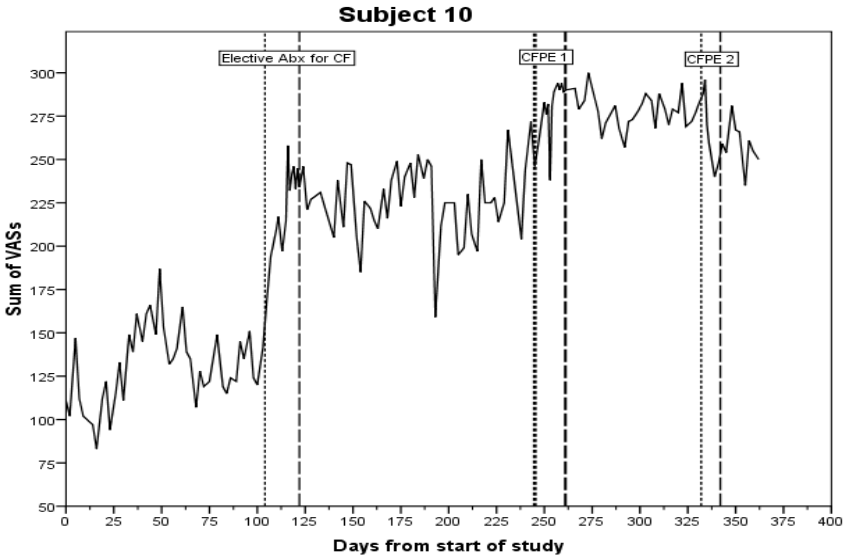

Figure 4-22 Subject 10: Response Profile Plot for Sum of VASs.

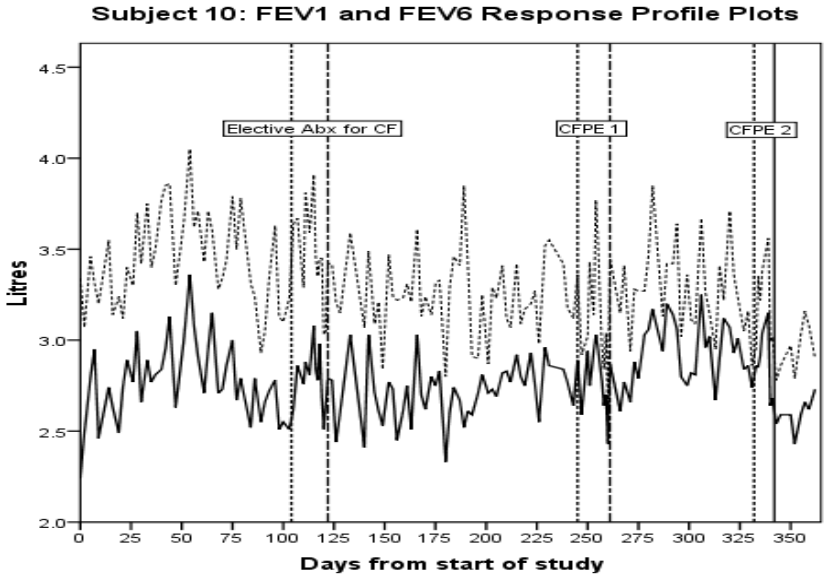

Figure 4-23 Subject 10: Response Profile Plot for FEV1 and FEV6

Subject 10		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.938****	**.943****	**.924****	**.968****	**.163***	**-.264****	**-.071**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**.041**	**.001**	**.379**
	N.	160	**160**	**159**	**160**	**159**	**158**	**157**	**156**
Cough VAS	rho	.938**	1.000	**.951****	**.931****	**.977****	**.165***	**-.283****	**-.040**
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**.038**	**<.001**	**.620**
	N.	160	160	**159**	**160**	**159**	**158**	**157**	**156**
Sputum VAS	rho	.943**	.951**	1.000	**.956****	**.985****	**.185***	**-.249****	**-.094**
	p value	<.001	<.001	.	**<.001**	**<.001**	**.020**	**.002**	**.246**
	N.	159	159	159	**159**	**159**	**157**	**156**	**155**
GWB VAS	rho	.924**	.931**	.956**	1.000	**.973****	**.222****	**-.244****	**-.069**
	p value	<.001	<.001	<.001	.	**<.001**	**.005**	**.002**	**.394**
	N.	160	160	159	160	**159**	**158**	**157**	**156**
Sum of VASs	rho	.968**	.977**	.985**	.973**	1.000	**.186***	**-.260****	**-.076**
	p value	<.001	<.001	<.001	<.001	.	**.020**	**.001**	**.350**
	N.	159	159	159	159	159	**157**	**156**	**155**
FEV1	rho	.163*	.165*	.185*	.222**	.186*	1.000	**.589****	**.039**
	p value	.041	.038	.020	.005	.020	.	**<.001**	**.626**
	N.	158	158	157	158	157	159	**158**	**155**
FEV6	rho	-.264**	-.283**	-.249**	-.244**	-.260**	.589**	1.000	**.010**
	p value	.001	<.001	.002	.002	.001	<.001	.	**.901**
	N.	157	157	156	157	156	158	158	**154**
Temp.	rho	-.071	-.040	-.094	-.069	-.076	.039	.010	1.000
	p value	.379	.620	.246	.394	.350	.626	.901	.
	N.	156	156	155	156	155	155	154	157

Table 4-12 Subject 10: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.11 Subject 11

Subject 11 had three treated CFPEs. The first two were treated with oral antibiotics, the third with IV antibiotics. There was a clear decline in symptoms leading up to the start of treatment for the first CFPE, over a period of around 50 days. This was associated with a decline in FEV1 from around 2.4 to around 2.0L. There followed a small response in symptoms and spirometry during the treatment. However this was not sustained and both symptoms and spirometry worsened a little after cessation of this first course of antibiotics. Two further courses of antibiotics followed, with good improvement in symptoms during the third course, which was of IV antibiotics. However, within 50 days of cessation

of IV antibiotics, symptoms had again worsened, without change in spirometry, suggestive of a pseudo-exacerbation. Symptoms did eventually improve without additional treatment, but never returned to the nadir seen prior to the first CFPE. Spirometry, however, gradually improved after the end of the last course of antibiotics, and by the end of the year, was close to values at the start.

Overall, subject 11 appears to have very variable symptoms that only partially responded to treatments for CFPEs. There was at least one and possible more pseudo-exacerbations. Table 4-13 below shows the correlations between the various components of the CMF. FEV1 has the strongest negative correlation with the General Wellbeing VAS (rho -0.348, $p<0.001$). There are moderately good positive correlations between the individual VASs, which improve when combined in the Sum of VAS score. The values for rho (0.392 to 0.656 are lower than for a number of other subjects. Temperature has no significant correlations.

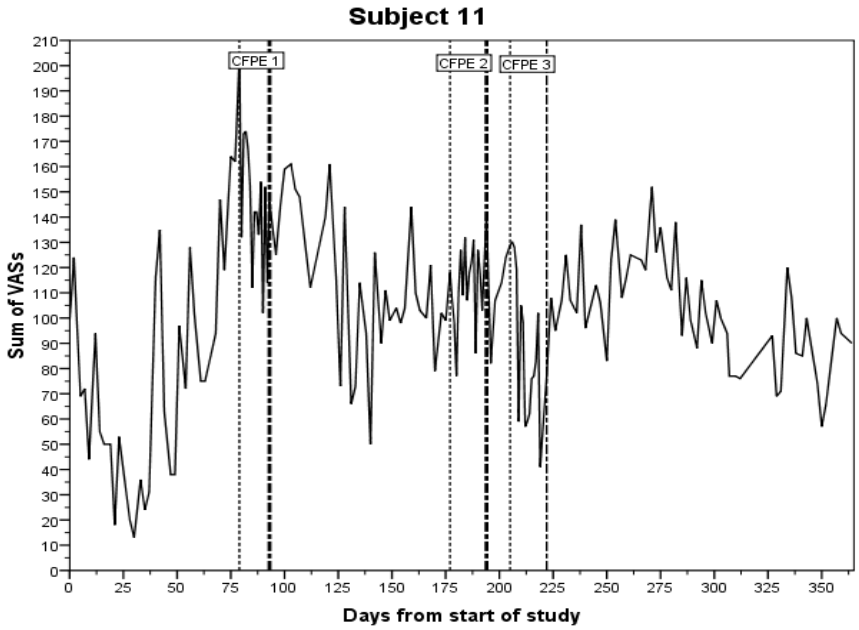

Figure 4-24 Subject 11: Response Profile Plot for Sum of VASs

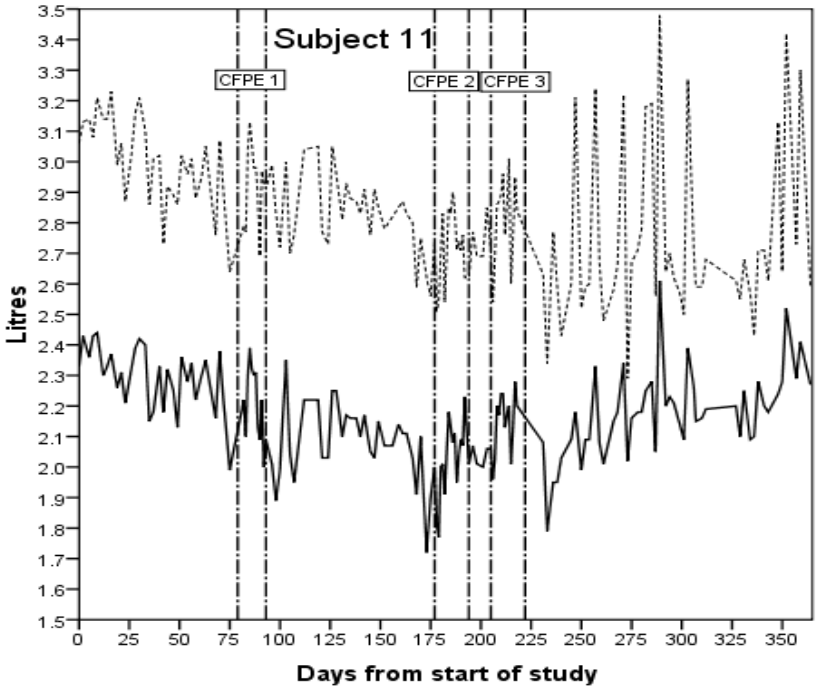

Figure 4-25 Subject 11: Response Profile Plot for FEV1 and FEV6

Subject 11		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.576****	**.392****	**.619****	**.836****	**-.321****	**-.160***	**.016**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.045**	**.835**
	N.	168	**168**	**168**	**168**	**168**	**159**	**159**	**168**
Cough VAS	rho	.576**	1.000	**.656****	**.478****	**.795****	**-.195***	**-.145**	**-.070**
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**.014**	**.069**	**.364**
	N.	168	168	**168**	**168**	**168**	**159**	**159**	**168**
Sputum VAS	rho	.392**	.656**	1.000	**.342****	**.690****	**-.179***	**-.220****	**-.082**
	p value	<.001	<.001	.	**<.001**	**<.001**	**.024**	**.005**	**.293**
	N.	168	168	168	**168**	**168**	**159**	**159**	**168**
GWB VAS	rho	.619**	.478**	.342**	1.000	**.764****	**-.348****	**-.127**	**.126**
	p value	<.001	<.001	<.001	.	**<.001**	**<.001**	**.112**	**.104**
	N.	168	168	168	168	**168**	**159**	**159**	**168**
Sum of VASs	rho	.836**	.795**	.690**	.764**	1.000	**-.315****	**-.170***	**.028**
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**.032**	**.715**
	N.	168	168	168	168	168	**159**	**159**	**168**
FEV1	rho	-.321**	-.195*	-.179*	-.348**	-.315**	1.000	**.701****	**-.039**
	p value	<.001	.014	.024	<.001	<.001	.	**<.001**	**.626**
	N.	159	159	159	159	159	159	**159**	**159**
FEV6	rho	-.160*	-.145	-.220**	-.127	-.170*	.701**	1.000	**.022**
	p value	.045	.069	.005	.112	.032	<.001	.	**.780**
	N.	159	159	159	159	159	159	159	**159**
Temp.	rho	.016	-.070	-.082	.126	.028	-.039	.022	1.000
	p value	.835	.364	.293	.104	.715	.626	.780	.
	N.	168	168	168	168	168	159	159	168

Table 4-13 Subject 11: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.12 Subject 12

Subject 12 was a student from the European Union studying at a local higher education facility. As such he retuned home during each of the academic holiday periods of Christmas, Easter and summer, and thus sampling ceased during these periods.

Subject 12 received treatment for four CFPEs during the study period. There were additional treated CFPEs whilst away from the UK during holiday periods, but these were not recoded. The first and fourth exacerbations were treated with IV antibiotics, whilst the second and third were treated with oral antibiotics.

In general Subject 12's VAS scoring was low, particularly when taking his pulmonary disease severity in to consideration. For the first exacerbation, which started 13 days after entry in to the study, a high Sum of VASs score can be noted, which appears to improve with treatment. However, the exacerbation occurred too close to the start of the study to allow a period of stability to be seen, if one existed. There was a marked initial response to the start of treatment, but symptoms worsened again before the end of treatment and remained at this level for about 14 days. There were also changes in spirometry during this time. However, although small, the changes ran counter to that which would be expected with a CFPE, with a fall during treatment and a rise after the end of treatment. The second and third treated CFPEs were associated with more modest changes in symptoms, without associated changes in spirometry. The fourth treated CFPE was immediately on returning from overseas, and it is thus difficult to comment accurately on this time. After a long summer holiday abroad, symptoms seemed to have worsened considerably on his return, with lung function considerably below that seen at the start of the study. This may constitute a pseudo-exacerbation.

Correlations between the components of the CMF are presented in Table 4-14 below. There was no correlation between any VAS value and lung function. The only correlation between an objective and a subjective measure was a weak negative correlation between FEV1 and temperature (rho -0.284, p=0.002). Correlations between separate VAS components were moderately strong and made stronger when combined in the Sum of VAS score.

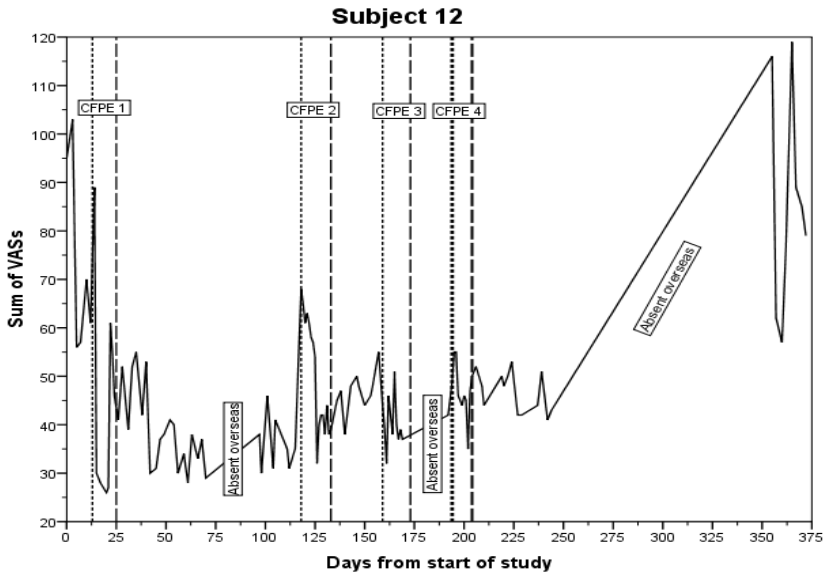

Figure 4-26 Subject 12: Response Profile Plot for Sum of VASs

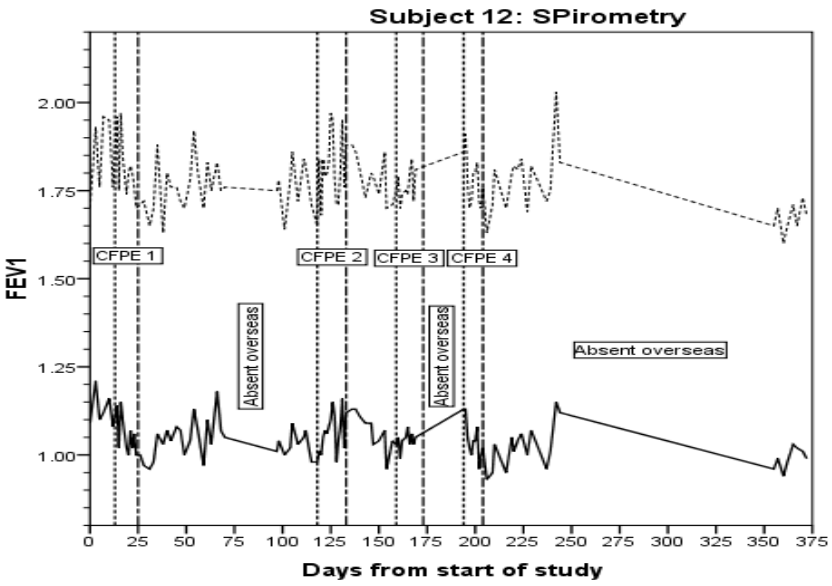

Figure 4-27 Subject 12: Response Profile Plot for FEV1 and FEV6

Subject 12		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.705****	**.613****	**.537****	**.844****	**-.118**	**-.084**	**.087**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**.214**	**.377**	**.358**
	N.	114	**114**	**114**	**114**	**114**	**113**	**113**	**114**
Cough VAS	rho	.705**	1.000	**.682****	**.583****	**.850****	**-.055**	**-.130**	**-.017**
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**.560**	**.170**	**.861**
	N.	114	114	**114**	**114**	**114**	**113**	**113**	**114**
Sputum VAS	rho	.613**	.682**	1.000	**.688****	**.865****	**-.069**	**-.139**	**.055**
	p value	<.001	<.001	.	**<.001**	**<.001**	**.470**	**.142**	**.559**
	N.	114	114	114	**114**	**114**	**113**	**113**	**114**
GWB VAS	rho	.537**	.583**	.688**	1.000	**.794****	**-.061**	**-.076**	**.060**
	p value	<.001	<.001	<.001	.	**<.001**	**.523**	**.424**	**.525**
	N.	114	114	114	114	**114**	**113**	**113**	**114**
Sum of VASs	rho	.844**	.850**	.865**	.794**	1.000	**-.084**	**-.129**	**.073**
	p value	<.001	<.001	<.001	<.001	.	**.376**	**.172**	**.438**
	N.	114	114	114	114	114	**113**	**113**	**114**
FEV1	rho	-.118	-.055	-.069	-.061	-.084	1.000	**.795****	**-.284****
	p value	.214	.560	.470	.523	.376	.	**<.001**	**.002**
	N.	113	113	113	113	113	114	**114**	**114**
FEV6	rho	-.084	-.130	-.139	-.076	-.129	.795**	1.000	**-.176**
	p value	.377	.170	.142	.424	.172	<.001	.	**.061**
	N.	113	113	113	113	113	114	114	**114**
Temp.	rho	.087	-.017	.055	.060	.073	-.284**	-.176	1.000
	p value	.358	.861	.559	.525	.438	.002	.061	.
	N.	114	114	114	114	114	114	114	115

Table 4-14 Subject 12: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05

4.2.13 Subject 13

Subject 13 generally scored his VASs at the very low end. He reported memories that as a young child he had been kept in hospital for a long period of time on oxygen and remembered severe breathlessness at that time. All breathlessness experienced since that time was mild by comparison.

Subject 13 had four treated CFPEs during the study period. All four were treated with IV antibiotics. Despite the low level of VAS scoring all four episodes were associated with discernible worsening symptoms, and subsequent response to treatment. At least three of the four were also clear associated with a decline in lung function that improved with treatment. There

were no clear pseudo-exacerbations, although there was a period at the very start of the study associated with higher symptom scores that resolved without recourse to antibiotic therapy. However, values were high at the start of the study, and thus no worsening was recoded, only an improvement, and thus cannot be called a pseudo-exacerbation.

Unlike the previous subject, subject 13 had good correlations between individual VAS scores and FEV1, with three of the four correlations being at rho~0.500. There was also a weak positive correlation between temperature and Sputum VAS and Sum of VASs. There was generally good correlation, except for General Wellbeing, which had lower correlations with the others than was seen for any other subject.

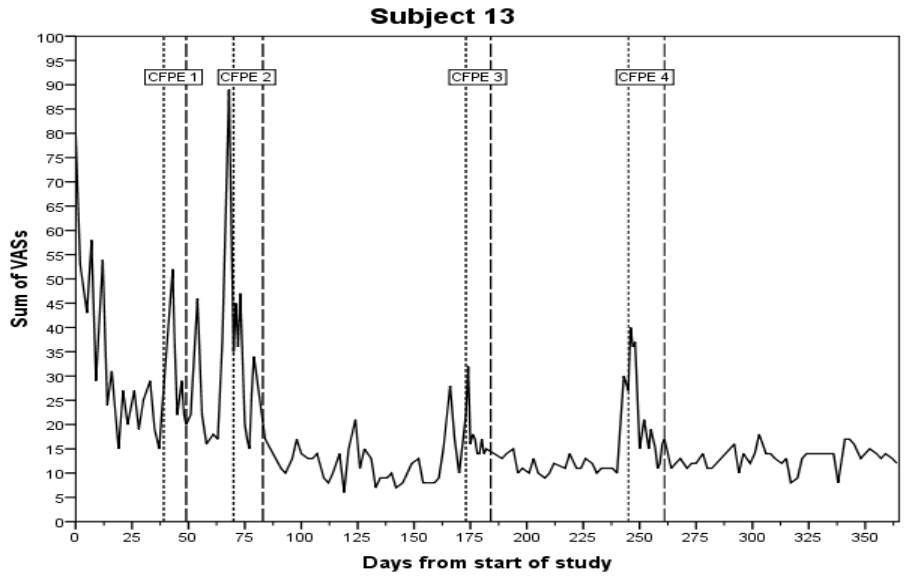

Figure 4-28 Subject 13: Response Profile Plot for Sum of VASs

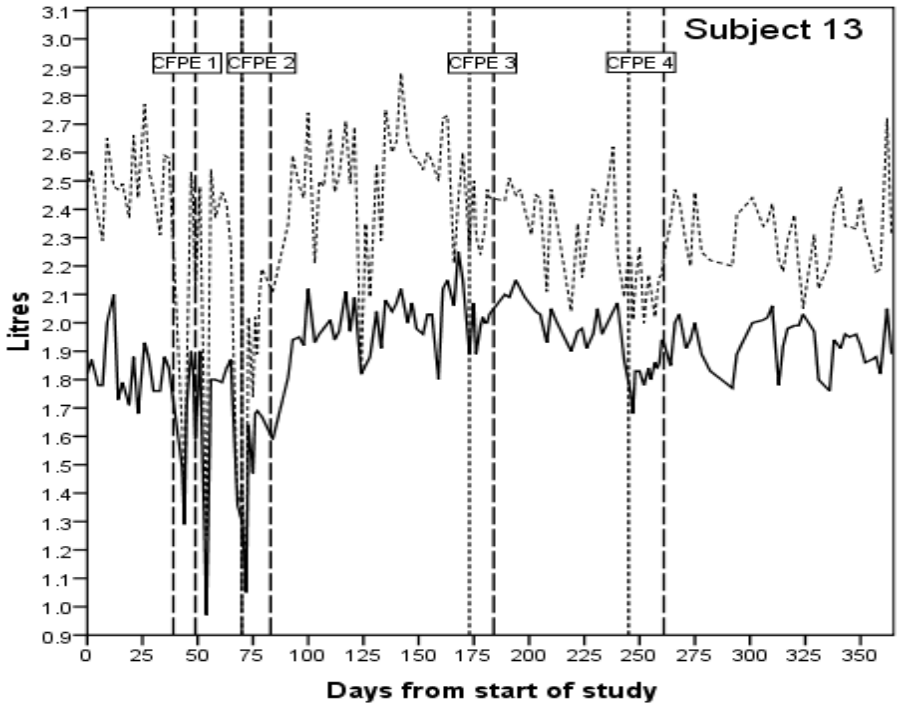

Figure 4-29 Subject 13: Response Profile Plot for FEV1 and FEV6

Subject 13		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.755****	**.656****	**.360****	**.886****	**-.494****	**-.256****	**.189***
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.001**	**.017**
	N.	160	**160**	**160**	**160**	**160**	**153**	**153**	**160**
Cough VAS	rho	.755**	1.000	**.758****	**.280****	**.874****	**-.512****	**-.230****	**.255****
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**.004**	**.001**
	N.	160	160	**160**	**160**	**160**	**153**	**153**	**160**
Sputum VAS	rho	.656**	.758**	1.000	**.315****	**.840****	**-.537****	**-.247****	**.289****
	p value	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**.002**	**<.001**
	N.	160	160	160	**160**	**160**	**153**	**153**	**160**
GWB VAS	rho	.360**	.280**	.315**	1.000	**.522****	**-.271****	**-.528****	**.078**
	p value	<.001	<.001	<.001	.	**<.001**	**.001**	**<.001**	**.328**
	N.	160	160	160	160	**160**	**153**	**153**	**160**
Sum of VASs	rho	.886**	.874**	.840**	.522**	1.000	**-.565****	**-.329****	**.280****
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**<.001**	**<.001**
	N.	160	160	160	160	160	**153**	**153**	**160**
FEV1	rho	-.494**	-.512**	-.537**	-.271**	-.565**	1.000	**.596****	**-.127**
	p value	<.001	<.001	<.001	.001	<.001	.	<.001	**.117**
	N.	153	153	153	153	153	153	**153**	**153**
FEV6	rho	-.256**	-.230**	-.247**	-.528**	-.329**	.596**	1.000	**.024**
	p value	.001	.004	.002	<.001	<.001	<.001	.	**.770**
	N.	153	153	153	153	153	153	153	**153**
Temp.	rho	.189*	.255**	.289**	.078	.280**	-.127	.024	1.000
	p value	.017	.001	<.001	.328	<.001	.117	.770	.
	N.	160	160	160	160	160	153	153	160

Table 4-15 Subject 13: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.2.14 Subject 14

Subject 14 had four treated CFPEs through the year. All four were treated with IV antibiotics. It is worth noting that the fourth and final CFPE was diagnosed and treated on the basis of haemoptysis, rather than on any reported deterioration in symptoms that could have been picked up with the VAS domains of the CMF. In general FEV6 values were very close, and often identical to FEV1 values, suggesting incomplete effort. However, there were changes seen in lung function that corroborated the changes in symptoms.

The worst symptoms were recoded at the start of the first CFPE, and these very rapidly improved with treatment. However this first CFPE was very quickly followed by a pseudo-exacerbation at around days 35 - 45, with high symptoms scores that exceeded any values seen throughout the remainder of the study. This first pseudo-exacerbation was also associated with some of the lowest lung function values during the study period. Following this first pseudo-exacerbation, there followed a second pseudo-exacerbation of less magnitude, also associated with a small but discernible decline in lung function. At day 123 there started treatment for the second CFPE, with a worsening in symptoms and lung function over the preceding 14 days. Notably, FEV1 declined from around 2.4L to 1.9L. Again there was good improvement with treatment.

Following the second CFPE, there followed a slow decline in lung function, associated with stable symptom scores. The third CFPE followed a similar pattern to the second. The final CFPE, as noted above, was for haemoptysis rather than other symptoms, and consequently there was no noticeable change in symptoms at that time.

Table 4-16 shows the correlations between the various components of the CMF. FEV1 correlated well with the subjective measures, with values for rho ranging from -0.445 to -0.392. FEV6 performed equally well if not better. There were also weakly significant weak positive correlations with temperature that were in a biologically implausible direction. The correlations of the VAS scores to each other were good, with the lowest rho value at 0.74. Combining the VAS scores in the Sum of VAS improved the correlation such that values of rho were all >0.900.

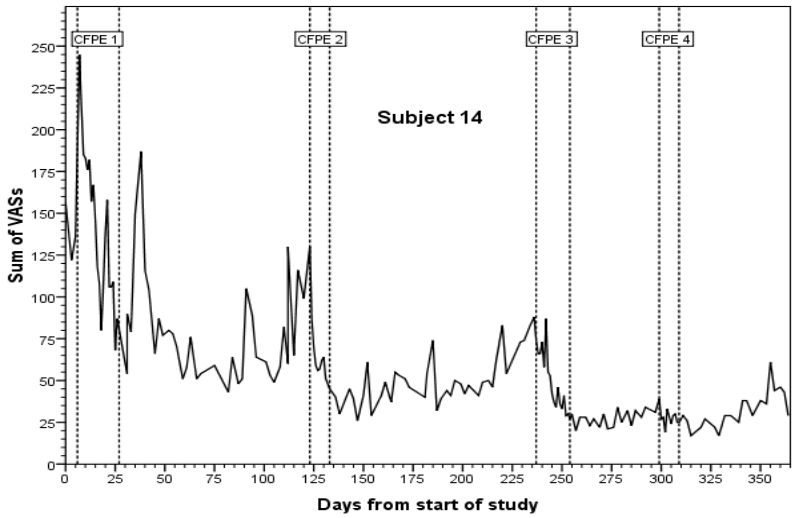

Figure 4-30 Subject 14: Response Profile Plot for Sum of VASs

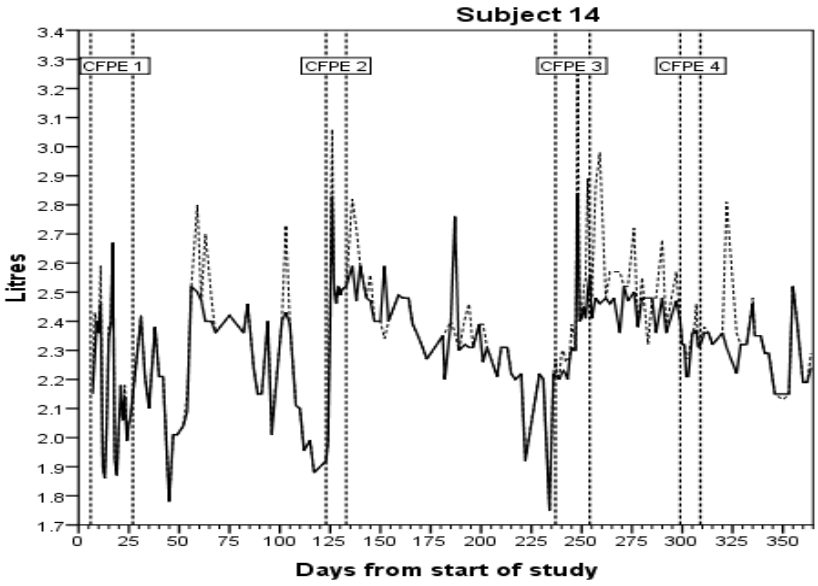

Figure 4-31 Subject 14: Response Profile Plot for FEV1 and FEV6

Subject 14		Breath VAS	Cough VAS	Sputum VAS	GWB VAS	Sum of VASs	FEV1	FEV6	Temp.
Breath VAS	rho	1.000	**.867****	**.833****	**.746****	**.901****	**-.392****	**-.409****	**-.108**
	p value	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.155**
	N.	174	**174**	**174**	**174**	**174**	**171**	**171**	**174**
Cough VAS	rho	.867**	1.000	**.953****	**.833****	**.978****	**-.397****	**-.417****	**-.047**
	p value	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**<.001**	**.536**
	N.	174	174	**174**	**174**	**174**	**171**	**171**	**174**
Sputum VAS	rho	.833**	.953**	1.000	**.865****	**.971****	**-.445****	**-.465****	**-.034**
	p value	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**<.001**	**.657**
	N.	174	174	174	**174**	**174**	**171**	**171**	**174**
GWB VAS	rho	.746**	.833**	.865**	1.000	**.900****	**-.442****	**-.461****	**-.068**
	p value	<.001	<.001	<.001	.	**<.001**	**<.001**	**<.001**	**.370**
	N.	174	174	174	174	**174**	**171**	**171**	**174**
Sum of VASs	rho	.901**	.978**	.971**	.900**	1.000	**-.437****	**-.460****	**-.055**
	p value	<.001	<.001	<.001	<.001	.	**<.001**	**<.001**	**.467**
	N.	174	174	174	174	174	**171**	**171**	**174**
FEV1	rho	-.392**	-.397**	-.445**	-.442**	-.437**	1.000	**.926****	**.175***
	p value	<.001	<.001	<.001	<.001	<.001	.	**<.001**	**.022**
	N.	171	171	171	171	171	171	**171**	**171**
FEV6	rho	-.409**	-.417**	-.465**	-.461**	-.460**	.926**	1.000	**.231****
	p value	<.001	<.001	<.001	<.001	<.001	<.001	.	**.002**
	N.	171	171	171	171	171	171	171	**171**
Temp.	rho	-.108	-.047	-.034	-.068	-.055	.175*	.231**	1.000
	p value	.155	.536	.657	.370	.467	.022	.002	.
	N.	174	174	174	174	174	171	171	174

Table 4-16 Subject 14: correlation between components of Clinical Monitoring Form, by Spearman's rank correlation. ** = correlations significantly different at p<0.05.

4.3 Relationship between Clinical Monitoring and CFPEs

It was clear from the above plots that the components of the CMF had a considerable variability, with many troughs and peaks unrelated to CFPEs or their treatment. Further analysis was therefore performed in order to establish whether fluctuations in the components of the CMF bore any relationship to the diagnosis of CFPE by the treating clinicians.

For each component of the CMF, a paired analysis was performed, of values around the time of a CFPE diagnosis with values from a period of stability in the 60 days preceding the CFPE. Values for the CFPE were taken as a mean of

the VAS or FEV1 values in the 7 days immediately prior to the start of treatment for a CFPE. Values for the period of stability were judged based on the appearances of the Sum of VASs response profile plot for that subject. More specifically, the period of stability was assessed as the lowest 7 day period in the 60 days prior to treatment for a CFPE, but not including the 7 days immediately prior to treatment. A CFPE was excluded if it occurred: within 21 days of a previous CFPE; within 21 days of the start of the study; or if there was insufficient data due to absence or incomplete CMFs. A period of stability could follow immediately on from a period of treatment for a CFPE, but could not include days when on treatment. Paired averages were compared using the paired student t test.

In total, there were 13 of 42 CFPEs that were excluded due to reasons outlined above. Results for the remaining 29 CFPEs (28 for FEV1 data) are presented below in Table 4-17 below. In general, all four VASs demonstrated a highly statistically significant difference between mean values at the time of exacerbation compared to during a period of preceding stability. The difference between the medians at the two time points varied from 8 to 14.7 VAS points, with p values ranging from <0.0001 for Breath, Cough and Sputum VASs to 0.002 for the General Wellbeing VAS. The difference in FEV1 was also significant ($p = 0.005$). Further details of the above findings can be seen in Figure 4-32 Figure 4-33 below. These two figures show that there is a clear deterioration in symptoms prior to a CFPE, and a commensurate improvement following the instigation of treatment. However, both the symptoms and lung function continue to improve following the end of the treatment period.

	Number of paired samples	Baseline median (IQR)	CFPE median (IQR)	*p* value
Br. VAS	29	13.7 (7-21)	21.7 (15-38)	<0.0001*
Cough VAS	29	16.0 (7-26)	28.0 (18-37)	<0.0001*
Sputum VAS	29	13.3 (8-26)	28.0 (19-34)	<0.0001*
GWB VAS	29	17.3 (9-26)	25.3 (18-42)	0.002*
FEV1 (L.)	28	1.85 (1.15-2.24)	1.76 (1.13-2.07)	0.005*

Table 4-17 Differences in median values of CMF components at baseline and at CFPE. (*= statistically significant at the 0.05 level Wilcoxon signed rank test)

In addition to the assessment of whether the individual components of the CMF could detect differences between periods of CFPE and stability, the time of the stable period prior to the start of treatment was recorded, using the same criteria above. Such analysis would cast light on the period of time where any aetiological agent causing a change in symptoms leading to a CFPE would be found, if such an agent exists).

As might be expected, the overall values vary widely. For the group as a whole there were 29 (of 42) analysable CFPEs, with a mean time of period of clinical stability of 24 days (±12) days prior to treatment for a CFPE.

Subject	No. of analysed exacerbations (No. missing)	Time of period of stability prior to CFPE in days (SD)
1	3 (0)	13 (3)
2	2 (1)	27 (1)
3	N/A	N/A
4	0 (3)	N/A
5	4 (1)	21 (11)
6	2 (0)	30 (7)
7	4 (0)	24 (15)
8	N/A	N/A
9	1 (4)	43
10	2 (0)	34 (2)
11	2 (1)	44 (17)
12	2 (2)	18 (5)
13	4 (0)	14 (9)
14	3 (1)	25 (8)
	Total = 29 (missing 13)	**Mean = 24 (12)**

Table 4-18 Last period of stability prior to CFPE, in days.

4.4 Summary of Clinical Monitoring and CFPE

In order to allow analysis of microbial changes before during and after CFPEs, a number of discrete CFPEs were selected for more detailed analysis. Only 12 of the 14 subjects had a CFPE event. One CFPE from each of these was selected, on the basis of temporal spacing away from other such events to allow each CFPE to be seen as discrete an event as possible. Results of bacterial community analysis can be seen in Chapter 6 - . Here are presented summary figures for the clinical monitoring during the CFPE that were selected for further bacterial community analysis.

There was no variation in mean oral temperature before during and after the 12 CFPEs. Values at start of treatment were 36.7^{o}C ($\pm$0.7), compared to 36.8^{o}C ($\pm$0.39) at 21 days prior to treatment; p= ns.

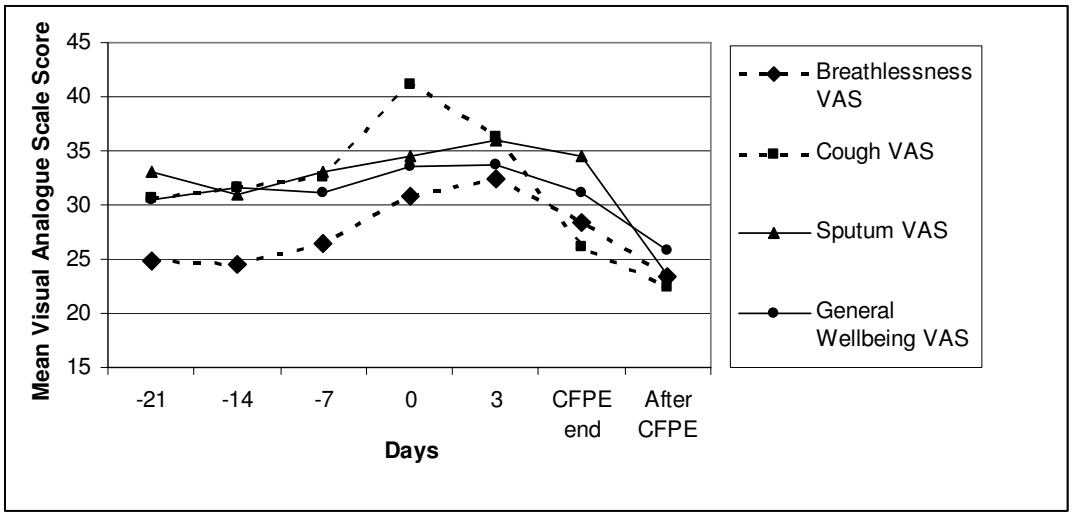

Figure 4-32 Summary of Visual Analogue Scale scores for 12 CFPEs from different subjects.

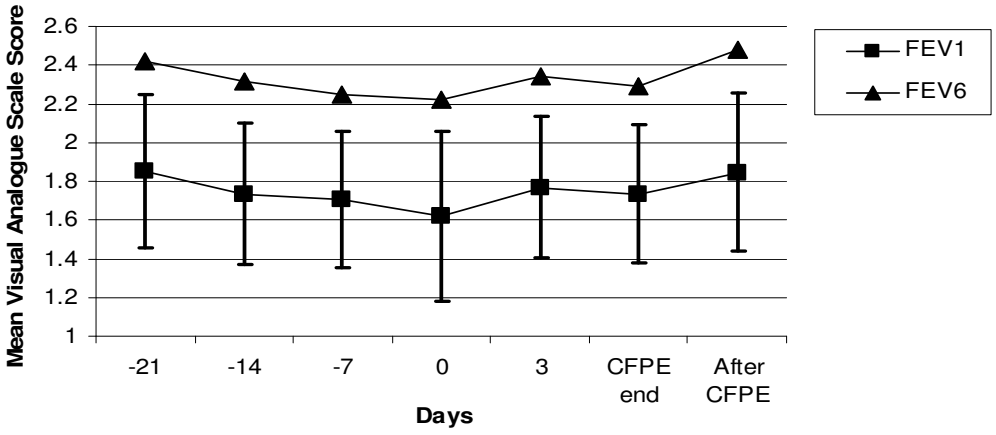

Figure 4-33 Changes in Lung Function Before During and After Treatment for 12 CFPE in 12 Subjects.

4.5 Discussion of Clinical Monitoring

Few longitudinal studies in respiratory illness have used Visual Analogue Scales to monitor symptoms (Marshall 2004; Rosenfeld *et al.* 2001). There are a number of simpler methods such as the general Likert Scale, or the Borg

Score (Borg 1982). VASs offer a much greater degree of freedom to subject, without imposition of artificially constraining metaphorical boxes (Gift 1989b). VASs have also been shown, in other clinical settings, to be more reproducible than either the Borg Score or the Likert Score for breathlessness (Grant *et al.* 1999), and to correlate to Peak Expiratory Flow Rate in Asthmatics (Gift 1989a). Grant *et al.* showed that a VAS is also the most sensitive to changes in breathlessness, as quantified by full cardio-pulmonary exercise testing (Grant *et al.* 1999). However, with this increased sensitivity to change, comes greater signal noise. As these set of VASs are partially unvalidated in this longitudinal setting, it is unproven as to what constitutes a Minimal Clinically Important Difference (MCID) on an individual basis. Ries *et al.* set out to determine an MCID for a breathlessness VAS and arrived at a figure of 10-20 mm. However, it would be flawed to impose this value on results presented here. First of all Ries *et al.* derived the value from interventional studies of Pulmonary Rehabilitation, with an intrinsic dichotomy that is lacking from the continuous variables studied here. Secondly, imposing an objective external MCID is counter-intuitive to the idea that a subject sets their own internal controls for what is important in determining the perception of a symptom. An example of this is provided by Subject 13 (see Section 4.2.13 above) who only had a total minimum to maximum range of 20 mm for Breathlessness VAS. Within this range there were four clear CFPEs all requiring intravenous antibiotics and three of four being associated with a decline in FEV1. If a MCID of 20 were imposed it is likely that no CFPEs would have reached the required threshold for a CFPE to be objectively diagnosed.

In addition to these concerns, the use of VASs makes inter-subject comparisons difficult. As the anchor phases are interpreted subjectively by subjects, they do not provide a means of quantifying changes observed between subjects. As can be seen from Figure 4-1 above the range of scores for any particular VAS varies greatly between subjects. If FEV1 percentage of predicted is used as a rough approximation of object breathlessness, it can be seen that there is little relationship between FEV1% and the range of VAS scores. For example, subject 10 has an FEV% of 86% predicted, but a median Breathlessness VAS of 60. This is in contrast to subject 12, who has an FEV1% of 30% predicted yet a lower median Breathless VAS of around 12. Despite this there has been shown to be a high degree of accuracy for the completion of VAS (see 2.7.1 above). In addition values for VASs are significantly higher in the week before treatment for a CFPE than in stable period in the preceding 60 days (see 4.3 above).

It can be seen from inspection of the Response Profile Plots for different subjects that changes in VAS appear to correlate better with the diagnosis and treatment of CFPE than in other subjects. For example, VAS RPPs for subject 10 do not appear to reflect the diagnosis and treatment of CFPEs. In contrast, symptom worsening is clearly documented for most if not all CFPEs for subjects 1, 5, and 14. The reasons for such discrepancies between subjects are not clear.

Thus, VASs appear to provide a good intra-subject record of symptoms, but a less good means of comparison between subjects.

Chapter 5 - Results: Periodic Sampling at Times of Clinical Stability

Bacterial diversity in CF airways has been demonstrated in previous studies (Bittar *et al.* 2008; Rogers *et al.* 2004). Some researchers have studied temporal changes in bacteria using culture independent techniques (Sibley *et al.* 2008g) over short periods focusing on particular species. No study however has taken a temporal approach to analysing if or how bacterial community diversity changes. Thus, it has not been possible to understand the significance of differences in the bacteria detected in different patients. Before studying CFPEs in relation to changes in the bacteria present, it was necessary to analyse the degree of flux in bacterial communities across time when subjects were clinically stable. Through a better understanding the fluctuations seen in bacterial communities over the whole study period, the fluctuations seen around periods of greatest clinical instability (CFPEs) could be put in context and interpreted.

In order to ensure that analysed samples came only from periods of stability, it was planned to analyse samples that were at least 30 days before the start, and 30 days after the end of systemic antibiotics. However, for some subjects that had more frequent exacerbations this was not possible. Table 5-1 below gives details of the times that the 'stable' samples were taken with respect to the start of the study and to periods of systemic antibiotics before and afterwards. The longest gap from 'Last systemic antibiotics' to analysed sputum sample was 1195 days (Subject 03, sample iv). The longest applicable gap from sample to 'Next systemic antibiotics' was 265 days (Subject 08, sample i; antibiotics given for unrelated non-pulmonary reason). Other subjects had no further recorded

systemic antibiotics. The shortest gap from 'Last systemic antibiotics' to analysed sputum sample was 5 days (Subject 05, sample ii). The shortest applicable gap from sample to 'Next systemic antibiotics' was 6 days (also Subject 05, sample iii). Due to the frequent CFPEs suffered by Subject 05, there were no samples which were more suitable for analysis.

Patient number	Sample	Day	Days Since Last Systemic Abx	Days until next Systemic Abx
1	i	5	> 30	81
	ii	194	32	44
	iii	296	39	n/a
	iv	345	88	n/a
2	i	64	49	29
	ii	157	51	97
	iii	304	35	n/a
	iv	360	56	n/a
3	i	15	851	n/a
	ii	122	958	n/a
	iii	243	1079	n/a
	iv	360	1196	n/a
4	i	1	41	175
	ii	103	115	73
	iii	318	57	32
5	i	13	?	52
	ii	111	5	16
	iii	148	12	6
	iv	190	22	26
	v	283	43	40
	vi	290	50	33
6	i	20	892	204
	ii	146	1018	65
	iii	288	26	n/a
	iv	351	89	n/a
7	i	27	218	149
	ii	137	342	20
	iii	218	57	20
	iv	363	28	n/a
8	i	10	> 30	265 (elective)
	ii	127	> 30	148 (elective)
	iii	255	> 30	21 (elective)
	iv	362	81 (elective)	n/a
9	i	5	18	128
	ii	44	35	97
	iii	100	91	39
	iv	307	27	30
10	i	19	31	89 (elective)
	ii	103	86	3 (elective)
	iii	234	97 (elective)	21
	iv	316	52	20
11	i	1	2989	79
	ii	129	35	49
	iii	255	32	n/a
	iv	365	142	n/a
12	i	69	33	50
	ii	238	69	21
	iii	371	166	n/a
	iv	504	299	n/a
13	i	1	267	39
	ii	139	55	35
	iii	297	35 (elective)	n/a
	iv	365	103 (elective)	n/a
14	i	64	36	59
	ii	167	32	71
	iii	204	69	34
	iv	356	46 (elective)	n/a

Table 5-1 Times of Sputum Sampling at Times of Clinical Stability, With Reference to Periods of Systemic Antibiotics. (Abx = antibiotics; N/A = not applicable).

It became apparent during preliminary analysis that the results were highly complex. It was noted that they shared similarities with ecological results from other non-clinical studies. Such studies have measured diverse communities (fish species in the Bristol Channel (Magurran & Henderson 2003)) and identified a division between species that are resident (core species) in the habitat in question, and a second group that can be identified as transient species (satellite species). Such techniques were applied to the data in this study in order to better understand the species.

In addition to core/satellite analysis, ecologists have used multivariate methods such as Principle Component Analysis to investigate complex data (Henderson & Seaby 2008). Others have applied such techniques to other clinical microbiological studies, of the bacterial community of the human gut for example (Engelbrektson *et al.* 2006), but not to Cystic Fibrosis respiratory samples.

5.1 Bacterial Clone Sequence Analysis

As outlined in Section 1.3.2 above, each culture independent molecular technique has its strengths and weaknesses. In brief T-RFLP is sensitive but does not provide an absolute species identification. However, it is relatively inexpensive and rapid, enabling the analysis of a large numbers of samples. There is also the theoretical risk that it may underestimate the number of species due to co-migration of two species with the same T-RF band length. However, it should be noted that this unlikely as there a median of 7.5 bands detected, spread over a possible range of more than 900 positions. In contrast, bacterial 16S clone sequence analysis is extremely specific, giving as exact a

speciation as is possible, but using current technologies, is extremely labour and cost intensive. In an attempt to improve the resolution of species/bands detected by T-RFLP during the longitudinal analysis, clone sequence analysis was employed on one sample from each subject. To some extent this allowed validation of the diversity detected by T-RFLP. It also allowed bands detected by T-RFLP to be assigned species names with a far greater degree of certainty, particularly in cases where the T-RFs of different species co-migrate to occupy the same band length position.

One sample from each of the 14 subjects was subjected to bacterial clone sequence analysis. Samples were chosen as close to the start of the study period as possible, at a time of clinical stability. A total of 2137 clones were analysed, with the number of clones per patient ranged from 90 to 258 (mean 153). The closest species matches to these sequences (at the level of 97% similarity) represented a total of 82 different bacterial species. These species were from four bacterial groups and 35 separate genera.

The number of different bacterial species identified per patient, as indicated by their closest species match, ranged from 2 to 42 (median 7.5; IQR 5.75-17.75)). It should, however, be noted that the similarity of the sequences to the published sequence data from particular bacterial species differed in many cases between clones. Further, in some cases the sequence similarity to the closest species match was as low as 82%, and 86% (*Alistipes shahii*, Sample 14 and *Barnesiella viscericola*, Sample 1, respectively). Given that many accept a genetic similarity level of 97% or greater to confer a species (Hey 2001), this would suggest that these are in fact new, previously undescribed species.

Sequences that most closely matched *P. aeruginosa* were detected in 13 of the 14 samples (the only sample in which it was not represented was Subject 01). *P. aeruginosa* clones represented a mean of 78.5% (SD±21.6) of the clones in samples in which it was detected, and represented the numerically dominant species in all of these 13 samples.

Of the other recognised CF pathogens (namely *S. aureus*, *H. influenzae*, *Burkholderia cepacia* complex, and *S. maltophilia (Gilligan 1991; Lyczak, Cannon, & Pier 2002)* only *S. aureus, H. influenzae* and *S. maltophilia* were detected in any of the cloned sequences, with each detected in one sample only. One sequence derived from *S. aureus* was detected in the sample from Subject 01, representing 0.78% of the sequences from that sample. Five sequences derived from *H. influenzae* were detected in the sample from Subject 14, representing 1.9% of the sequences from that sample. Four sequences derived from *S. maltophilia* were detected in the sample from Subject 01, representing 3.1% of the sequences from that sample.

The same samples were also subject to standard bacterial culture as described in Section 2.2.1 above, to allow comparison of species detected and identified. The results of selective bacterial culture are presented in Table 5-2. In 11 of the 13 subjects for whom results from the comparable sputum sample were available, *P. aeruginosa* was identified. From 10 of 13 subjects 'Oral flora' was detected. In 6 of the 13 subjects a fungus was detected. MRSA was identified in one subject.

Within the sequence set there were 16 genera, from four different Groups, that have not been reported in the literature as having been detected, either by culture-based analysis of molecular-based analysis, within CF lung infections.

The 17 species that this represents, the samples from which they were derived, and the number of clones per species, are shown in Table 5-2 below.

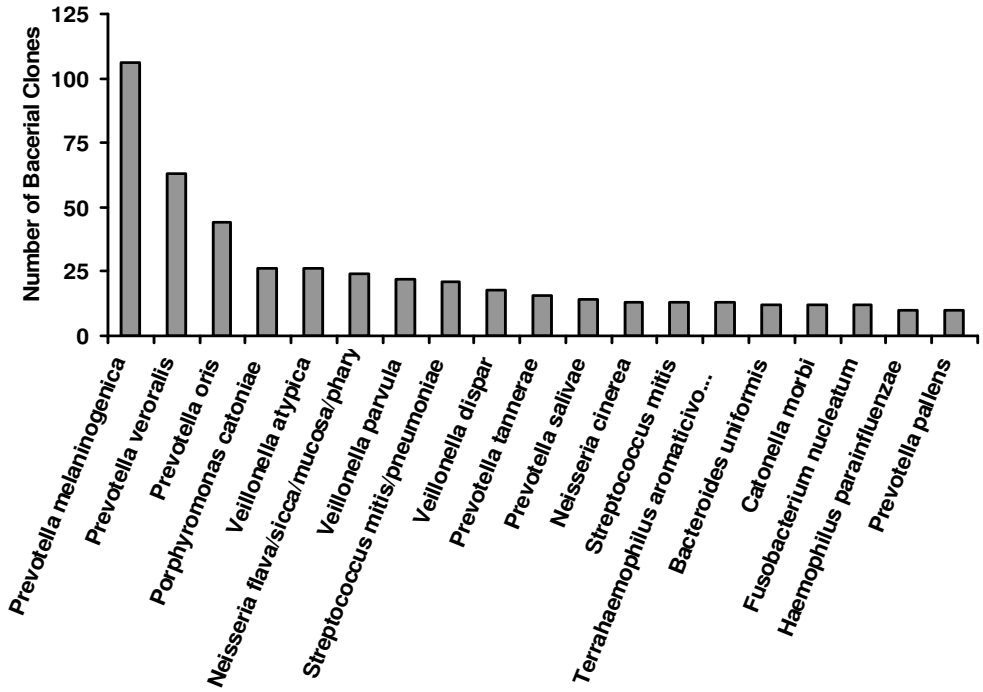

Figure 5-1 Rank abundance for species detected by bacterial clone sequence analysis (*P. aeruginosa* excluded: n=1510)

Species	Subject	Number of clones
Alistipes shahii	14	1
Alishewanella fetalis	8	1
Barnesiella viscericola	1	1
Catonella morbi	1	9
	6	2
Clostridium metallolevans	13	1
Dialister invisus	1	1
Dorea longicatena	12	1
Gemella haemolysans	5	2
	6	3
	14	1
Megasphaera micronuciformis	1	3
	6	4
Mogibacterium neglectum	1	2
Oribacterium sinus	6	1
Rothia dentocariosa, Rothia proteobacteria	9	1
	14	1
Selenomonas infelix	14	3
Tannerella forsythensis	1	1
Terrahaemophilus aromaticivorans	1	8
	6	5

Table 5 2 Bacterial species identified by clone sequencing that have not previously been reported in CF respiratory infections.

Subject	"Species" 1	"Species" 2	"Species" 3	Species 4
01	Oral Flora (+)			
02	*P. aeruginosa* (+++)	*Pseudomonas* spp. (+++)	Oral flora (++)	*Aspergillus fumigatus* (+++)
03	Oral Flora (+)	*P. aeruginosa* mucoid (++)	*Pseudomonas* spp. (++)	
04	MRSA (*S. aureus*) (+)	Oral flora (+++)		
05	*Pseudomonas* spp. (+++)	Oral flora (+++)	*P. aeruginosa* mucoid (+++)	*Aspergillus fumigatus* (+++)
06	*Pseudomonas* spp. (+++)	Yeasts (Scanty Growth)	Oral Flora (+++)	*P. aeruginosa* (+++)
07	*P. aeruginosa* (+++)	*P. aeruginosa* mucoid (+++)		
08	Pseudomonas spp. (+++)	*Aspergillus fumigatus* (+)	Yeasts (+)	Oral Flora (+++)
09	*Pseudomonas* spp. (+++)	*Pseudomonas* spp. (+++)	*Pseudomonas* spp. (+++)	Oral Flora (+++)
10	Data Missing			
11	*P. aeruginosa* (+++)	*Pseudomonas* spp. (+++)	Yeasts (+)	*Aspergillus fumigatus* (+)
12	*Pseudomonas* spp. (+++)	*Pseudomonas* spp. (+++)	*Pseudomonas* spp. (+++)	Oral Flora (+++)
13	*P. aeruginosa* mucoid (+++)	*Pseudomonas* spp. (+++)		
14	*Pseudomonas* spp. (+++)	*Pseudomonas* spp. (+++)	Oral Flora (+++)	

Table 5-2 Semi-quantitative bacterial culture result for samples also subjected to bacterial clone sequencing.

5.1.1 Classification of Bacteria Detected by Clone Sequence Analysis

5.1.1.1 *Aerobes vs. Anaerobes*

The full list and identity of bacterial clones has been included in the Appendix (Table 9-1 below). In summary there are a total of 82 species, 36 (44%) of which have been considered to be obligate anaerobes. The remaining species (n=46, 56%) can be classified as aerobes, which includes microaerophiles, facultative anaerobes and aerobes (Brenner *et al.* 2005)

5.1.1.2 *Bacteria Associated with the Human Oropharynx*

Bacterial clone sequence analysis allows accurate speciation of clones. In Appendix Table 9-1, it can be seen that 59 of 82 (72%) of identified bacterial species are associated with the human oropharynx based on an analysis of the literature available (Aas *et al.* 2005; Brenner *et al.* 2005; Gibbons *et al.* 1963; Igarashi *et al.* 2009; Sakamoto, Umeda, & Benno 2005). This includes species from the genera *Prevotella* , *Porphyromonas*, *Veillonella* and *Rothia*. Other bacterial groups have been associated with other parts of the human microbiota, such bacteria from the genus *Bacteroides*, which are associated with the normal human gastrointestinal tract flora (Brenner *et al.* 2005).

5.2 TRFLP Sampling Overview

5.2.1 TRFLP

Results of periodic analysis of samples from each subject at periods outside of treatment for CFPEs are presented below. The mean bacterial species richness by T-RFLP compared to species detected by culture are presented in **Figure 5-4** and Table 5-4. Across the four time points in the study, the mean

number of species detected by T-RFLP ranged between 5.4 species (SD±2.5) and 6.4 species (SD±2.7). Mean bacterial species richness as detected by culture varied from 1.6 to 2.0 species (SD±0.49-0.67). Using the unpaired student t test, differences in the means for species richness by T-RFLP and culture was highly statistically significant ($p<0.0001$) at all time points. In addition to this difference, there was no correlation between the number of species detected by culture and the number of species detected by T-TFLP (Spearman's *rho* 0.675; *p*=NS).

In order to assess the similarity between consecutive samples, the Sorensen Index of Similarity was used as described in Section 2.5 above. The results for both T-RFLP and culture are presented, for all subjects in Figure 5-5 below. The lines representing the mean Sorensen Index for the 14 subjects at each time point for both culture and T-RFLP are not statistically different, even though the actual number of species detected by the two techniques was different (see above). This shows that the likelihood of two consecutive samples being bacteriologically similar are the same whether the means of testing is culture based or by T-RFLP.

The individual fluctuations in bacterial profiles by T-RFLP over the 12 months are presented Figure 5-2 and Figure 5-3 below. It can be seen that some subjects showed remarkable consistency, with species detected across long periods of time (e.g. subjects 1, 2, 3, 6, 7, 11, 13 and 14), despite courses of antibiotics for CFPEs in most cases, and long term antibiotic therapy in all. In others however, there is considerable flux of species across the time points (4, 5, 8, 9, 10 and 12). There is no correlation between disease severity and mean

Sorensen Index (Pearson correlation coefficient; data not shown), although the low numbers of subjects limits the power to detect a real difference.

Subject	Stable or Unstable	Most Dominant	Second Dominant	Third Dominant
1	S	Sp. 17	*T. forsythensis / A. shahii / C. gracilis*	Sp. 27
2	S	*P. aeruginosa*	*R. dentocariosa*	Sp. 39
3	S	*P. aeruginosa*	*R. dentocariosa*	*Porphyromonas. / Prevotella. / Bacteroides* sp.
4	U	*Prevotella* sp.	Sp. 17	Sp.1
5	U	*P. aeruginosa*	Sp. 17	*Prevotella mel.*
6	S	*P. aeruginosa*	*H. parainfluenzae*	*Megasphaera* m.
7	S	*P. aeruginosa*	*R. dentocariosa*	Sp. 23
8	S	*P. aeruginosa*	*Veillonella* spp.	*Prevotella* spp.
9	U	T-RF 212	*P. aeruginosa*	*H. parainfluenzae*
10	U	*P. aeruginosa*	*H. parainfluenzae*	*H. parahaemolyticus*
11	S	Sp.17	*T. forsythensis / A. shahii / C. gracilis*	*P. aeruginosa*
12	U	Sp. 17	Sp. 19	Sp. 10
13	S	Sp. 17	*T. forsythensis / A. shahii / C. gracilis*	*P. aeruginosa*
14	S	Sp. 17	*P. catoniae*	*P. aeruginosa*

Table 5-3 Top three most dominant species/bands by T-RFLP. 'S' and 'U' = Stable and Unstable bacterial communities over time.

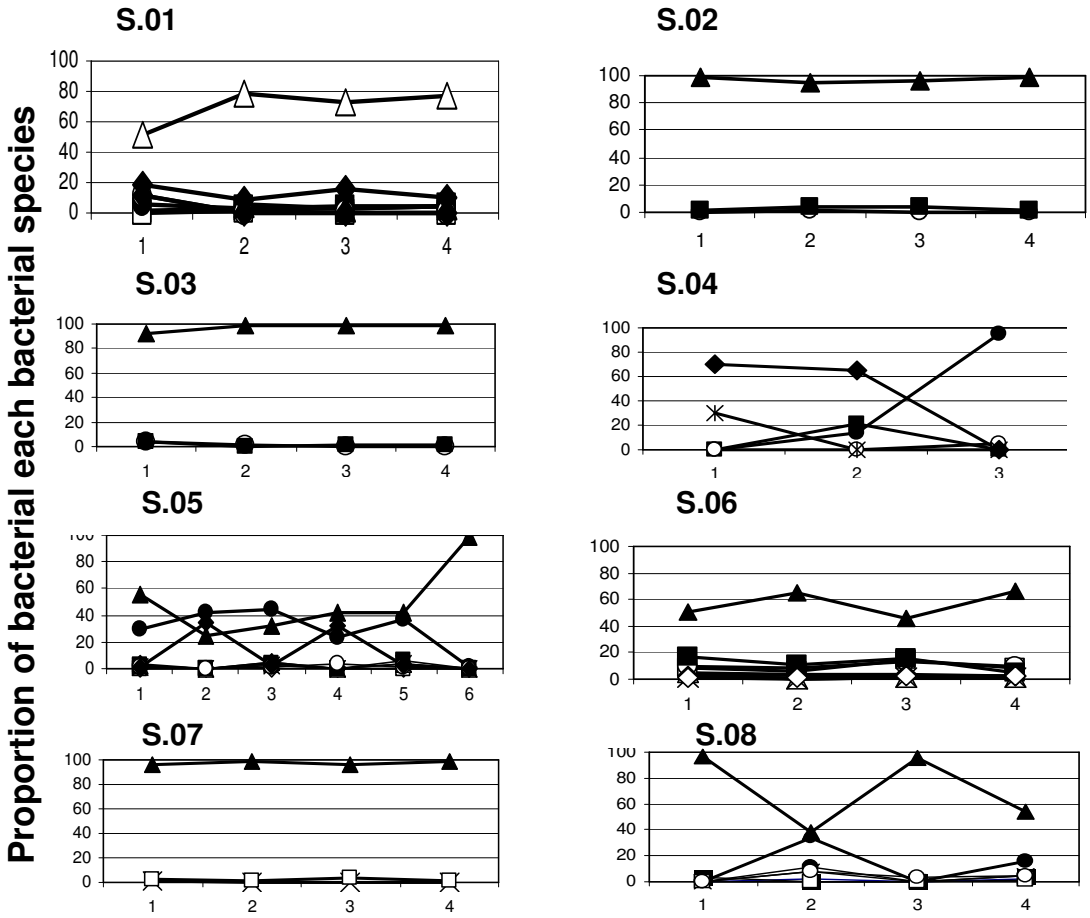

Figure 5-2 Bacterial Community Stability over Study Period (Time – X axis), for Subjects (S.) 01-08. Y axis is percentage of community. Black triangles represent percentage of *P. aeruginosa*. Values represent PMA treated T-RFLP results.

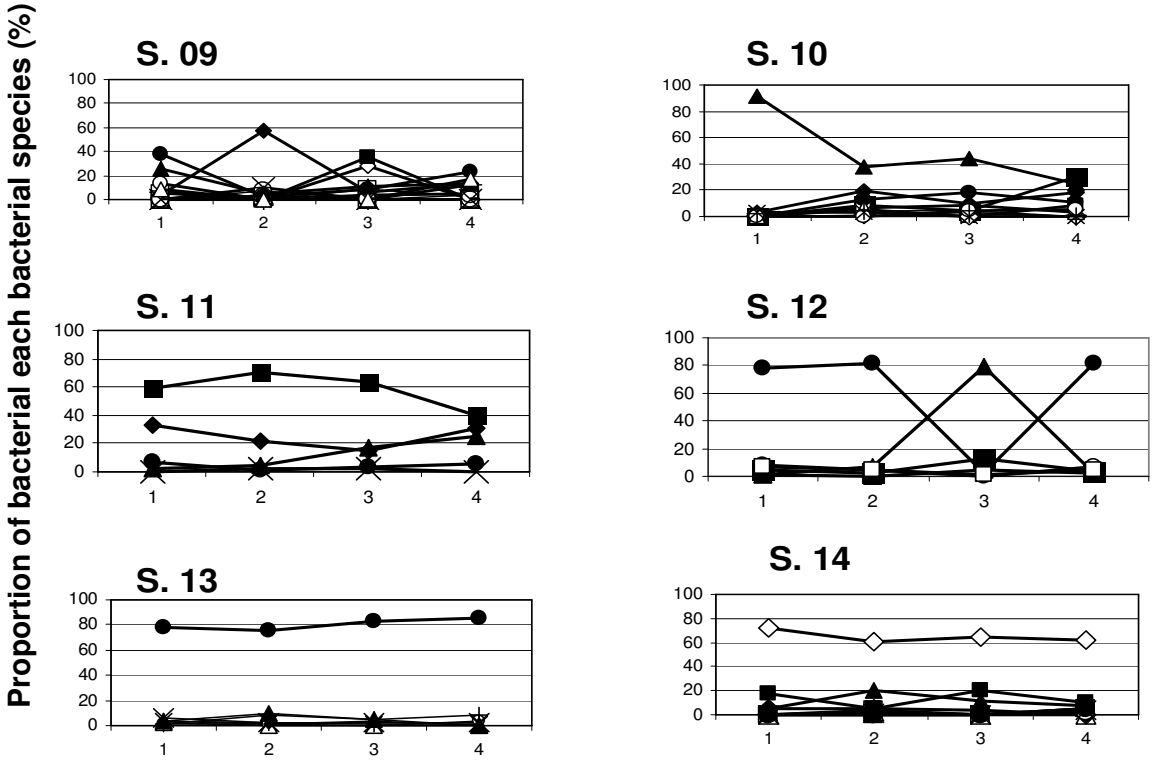

Figure 5-3 Bacterial Community Stability (DNA) over Study Period (Time – X axis), for Subjects (S.) 09-14. Y axis is percentage of community. Black triangles represent percentage of *P. aeruginosa*. Values represent PMA treated T-RFLP results.

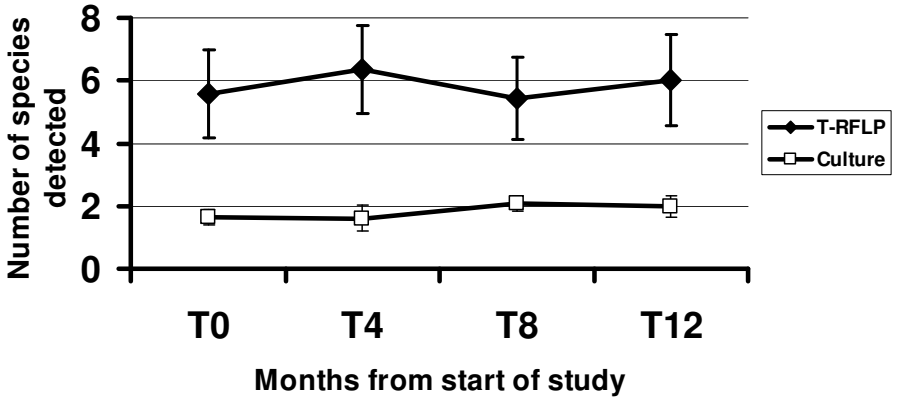

Figure 5-4 Mean bacterial species richness, measured by T-RFLP and culture. *p<0.0001 across all time points by unpaired student t test. Y-axis error bars represent 95% confidence intervals.

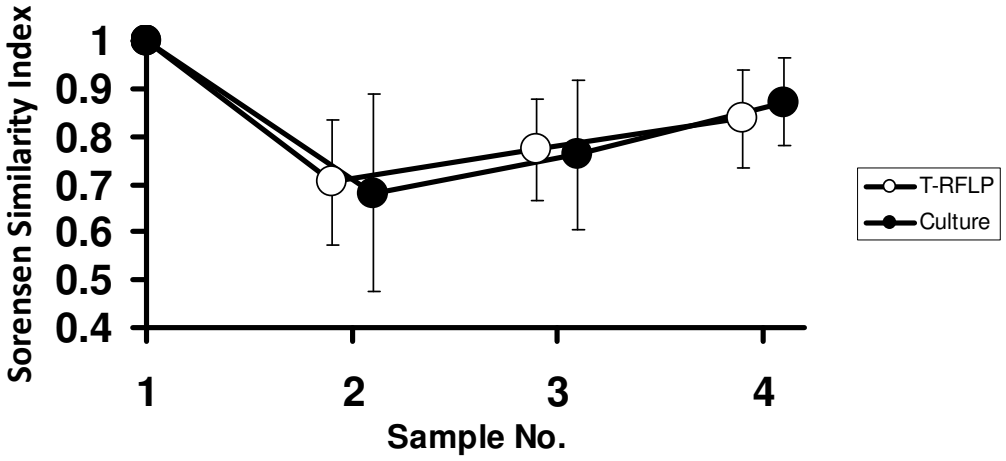

Figure 5-5 Similarity between bacteria in samples at four time points of clinical stability using Sorensen Index , by T-RFLP and culture. Y-axis error bars represent 95% confidence intervals. There was no significant difference between the methods of bacterial detection.

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Subject	Bacterial Species Richness		Sorensen Similarity Index	
	Culture	T-RFLP	Culture	T-RFLP
1	2.50	5.50	0.79	0.89
2	2.00	3.25	0.88	0.93
3	1.75	3.50	0.83	0.96
4	1.67	2.33	0.33	0.60
5	1.50	5.50	0.67	0.80
6	1.25	10.00	0.89	1.00
7	1.50	3.25	0.83	0.96
8	2.25	5.75	0.83	0.69
9	1.75	9.00	0.83	0.58
10	2.00	8.25	1.00	0.79
11	1.75	5.25	0.83	0.93
12	2.00	5.50	1.00	0.90
13	1.75	7.25	0.92	0.89
14	2.00	6.50	1.00	0.75
Total	**1.83***	**5.77***	**0.83**	**0.83**

Table 5-4 Species richness and similarity between samples during periods of clinical stability. *=statistically significant difference between the means by unpaired student t test ($p<0.001$).

5.2.2 Comparison of Bacterial Community Structure by Genotype

In order to investigate whether Cystic Fibrosis genotype exerted an influence on bacterial community structure, subjects were divided into those who were known to be homozygous for the F508 mutation, and those who were heterozygous for the F508 mutation, with the second allele being another, non-F508, mutation. The results are presented in Figure 5-6 below.

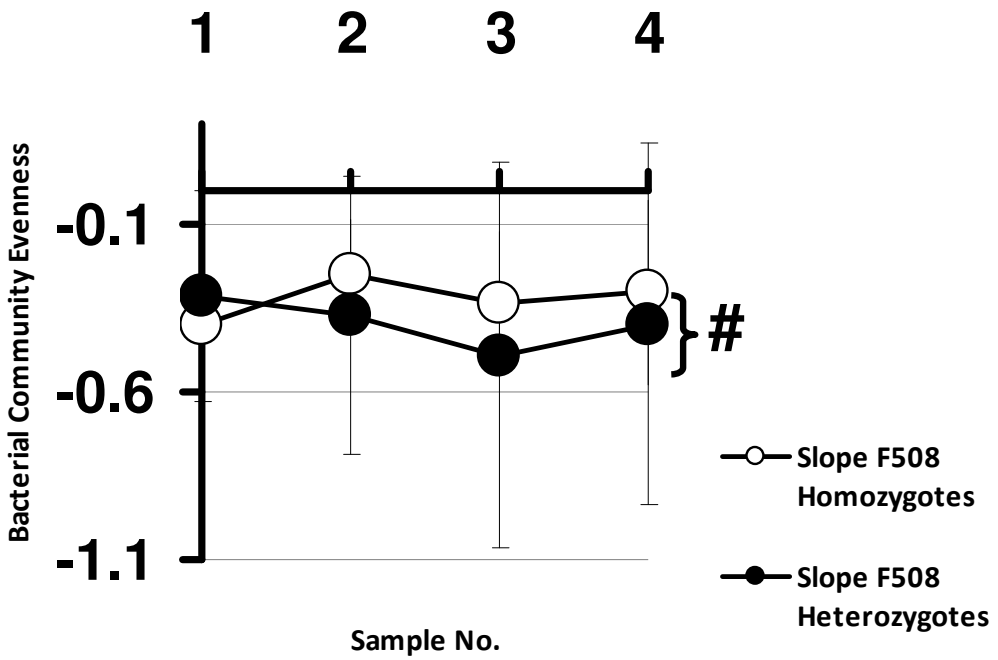


Figure 5-6 Comparison of bacterial community slope by CF genotype over four stable time points. # = p value for differences not significant.

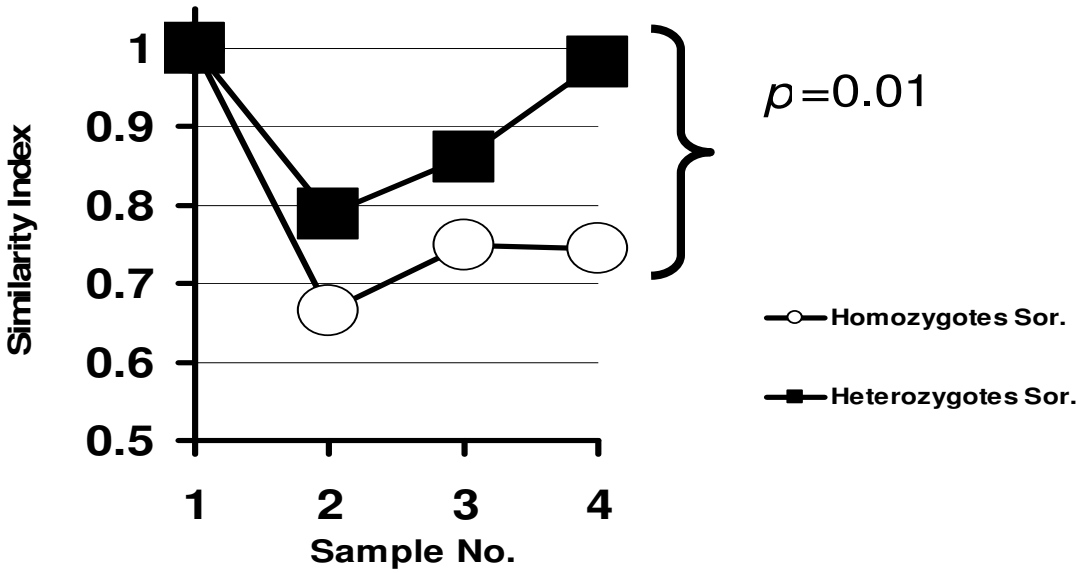


Figure 5-7 Comparison of bacterial community similarity by CF genotype, using Sorensen Index (=Sor.).

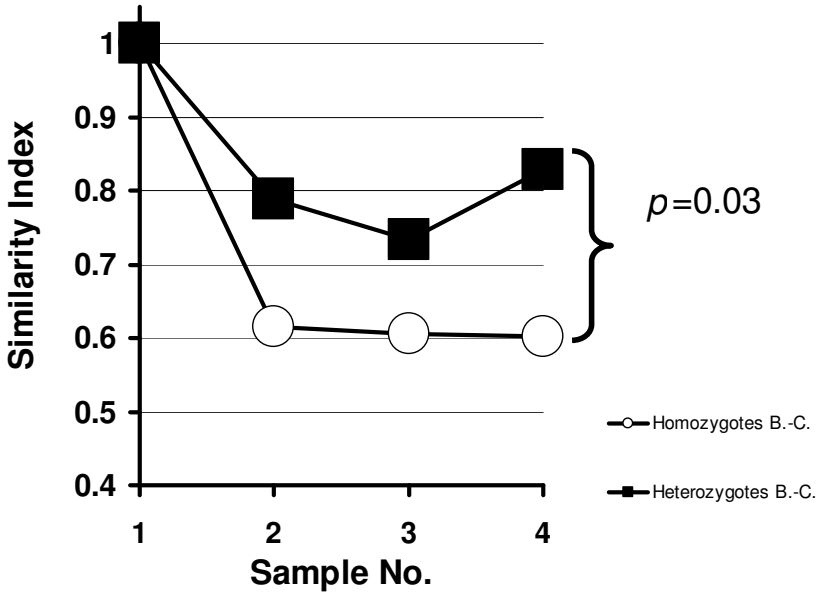


Figure 5-8 Bray-Curtis Similarity Index by CF genotype.

It can be seen that there are significant differences between heterozygotes and homozygotes by similarity indices. For heterozygotes and homozygotes, the calculated mean Sorensen Index 0.87 (±0.20), vs. 0.72 (±0.19) respectively

were statistically different (p=0.01) as were the comparable findings for the mean Bray-Curtis Index i.e. 0.78 (±0.18) vs. 0.60 (±0.29) respectively (p=0.03).

5.2.3 Comparison of Bacterial Community Structure by Lung Function

In order to determine if interplay between lung function and bacterial community structure, the 14 subjects were divided in to two groups on the basis of their stable lung function at the start of the study. The chosen cut off for lung function was an FEV1 of 50% of the predicted value. Using this classification two groups were 6 'Low' lung function (FEV1 <50% predicted), and 8 'High' lung function (FEV1 ≥50%). These groups are as shown in Table 5.8 with low lung function patients including subjects 3, 4, 5, 6, 9 and 12 and high function patients including subjects 1, 2, 7, 8, 10, 11, 13 and 14.

	Subject
Low Lung Function (FEV1 <50%)	3, 4 , 5 , 6 , 9 ,12
High Lung Function (FEV1 ≥50%)	1 , 2 , 7 , 8 , 10 , 11 , 13 , 14

Table 5-5 Division of Subjects by Lung Function during a period of clinical stability at the start of the study

The mean bacterial community slope was calculated for each of the four stable time points. In the low lung function group the mean (±SD) slope was 0.46 (±0.33), compared to a mean slope of -0.27 (±0.31) for the high lung function group (The results are presented in Figure 5-9 below). It can be seen that

subjects with lower (worse) lung function have a statistically significantly steeper bacterial community slope, with the difference between the two groups giving a *p* value of 0.02.

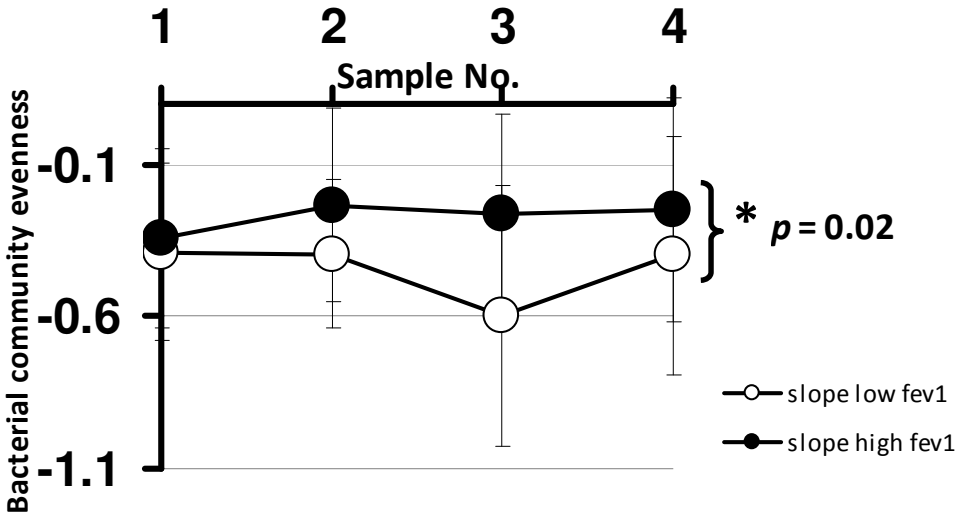


Figure 5-9 Comparison of bacterial community evenness by Lung Function. * p value = 0.02.

The relationship between similarity indices and lung function was assessed. As described in Methods Section (see 2.5 above) the Sorensen Index and the Bray-Curtis Index both measure the similarity between two ecological communities, using binary and quantification values respectively. The amount of flux between subjects with high and low lung function, as measured using both the Sorensen and Bray-Curtis Index, and presented in Figure 5-10 below. Sorensen Index mean (±SD) for Low FEV1 0.74 (±0.24) and for the High FEV1 group 0.82 (±0.17); *p*=NS. The Bray-Curtis Index was 0.58 (±0.34) for the Low FEV1 group, and 0.75 (±0.16) for the high FEV1 group (*p*= 0.036).

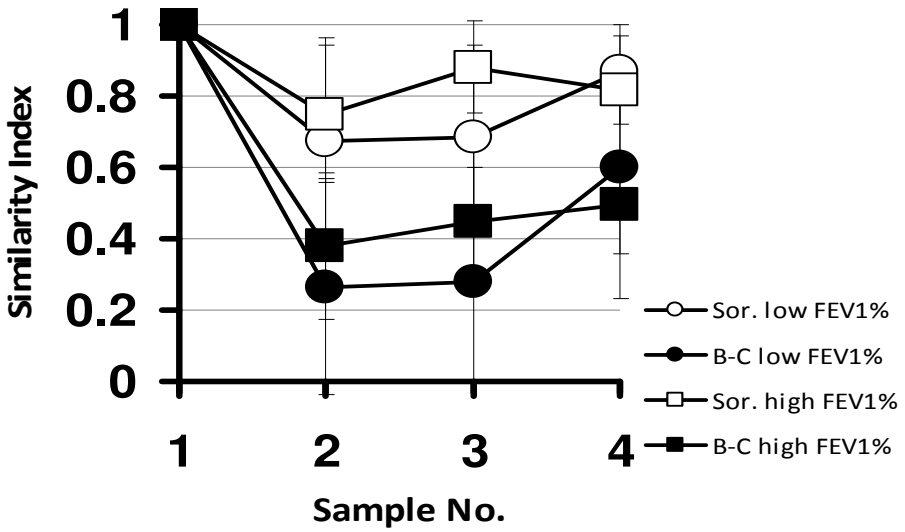


Figure 5-10 Similarity indexes by Lung Function. Sor.= Sorensen Index; B-C = Bray-Curtis Index. Difference between Sorensen Index = NS; diffierence between groups by Bray-Curtis Index *p* = 0.036, using unpaired t test.

One further analysis between the high and low lung function groups was undertaken. The results of the comparison of bacterial diversity between High and Low FEV1 groups are presented in Figure 5-11 below. The overall mean for the high lung function group was 6 (SD± 0.7) species per sample. The overall mean for the low lung function group was lower at 5.25 (±0.70) species per sample. The difference between the two groups was statistically significant (*p* = 0.048, unpaired t test).

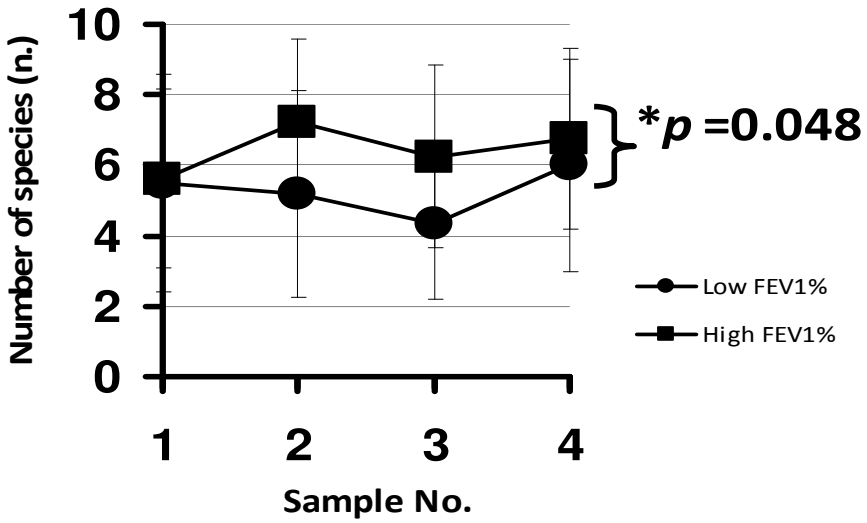


Figure 5-11 Species richness for 4 stable samples from each subject, separated by Lung Function. * *p* = 0.048, using unpaired t test.

5.2.4 Comparison of Bacterial Community Structure by Gender

In order to determine if there existed interplay between lung function and bacterial community structure, the 14 subjects were divided on the basis of their gender. There were 8 females and 8 males (see Section 3.1.1 above).

Results for bacterial community structure by gender are presented in Figure 5-12 below. The mean (+SD) slopes for females and males respectively were -0.48 ($\pm$0.04), and -0.26 ($\pm$0.05). This difference was statistically significant (p= 0.0004) using unpaired t test.

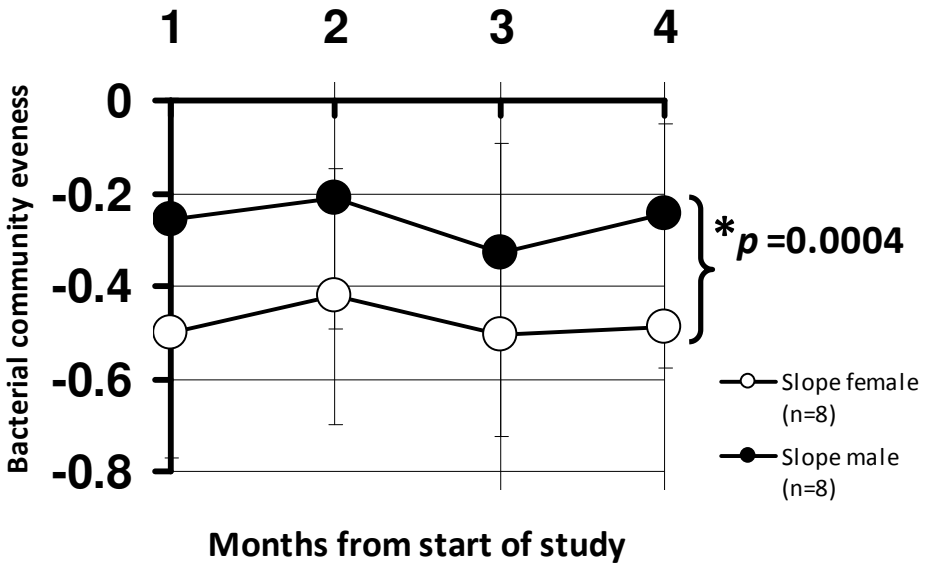


Figure 5-12 Bacterial community slope for 4 stable samples from each subject, separated by gender. * p = 0.0004, using unpaired t test. Error bars represent standard deviation.

Further assessment of the bacterial community differences between genders was done, using measures of similarity between consecutive samples.

Results separated by gender for the Similarity Indexes are presented in Figure 5-13 below. The mean values (±SD) for the Sorensen Index for females and males respectively are 0.79 (± 0.09) and 0.78 (±0.03); p = NS. The mean values (±SD) for the Bray-Curtis Index for females and males respectively are 0.72 (± 0.02) and 0.64 (±0.02); p = 0.01, using the unpaired t test.

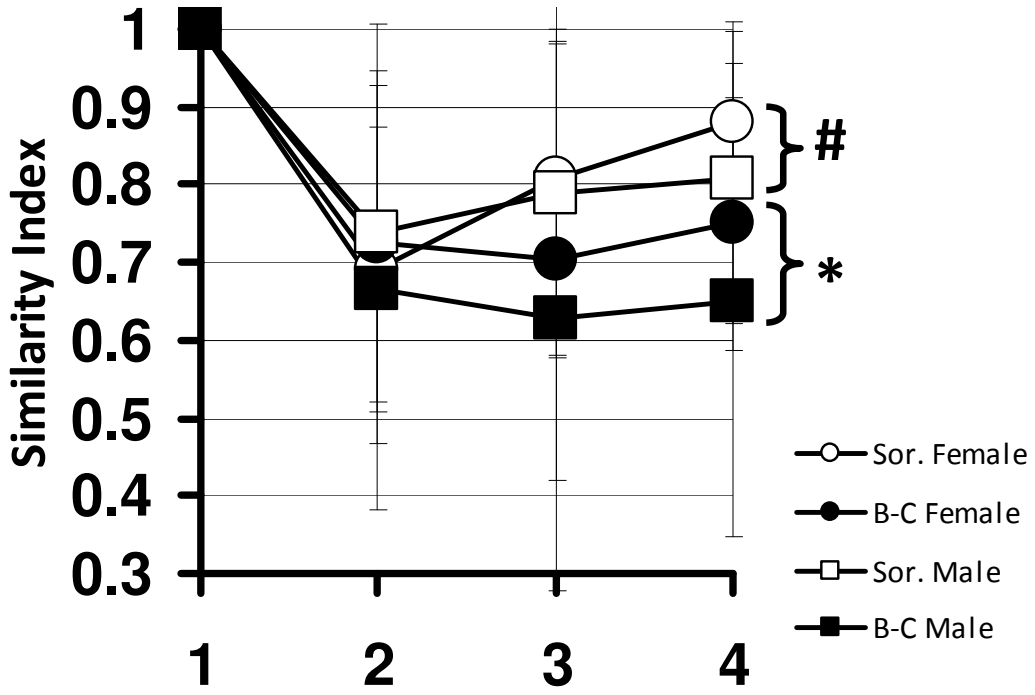


Figure 5-13 Similarity Indexes (Sor. = Sorensen Index; B-C = Bray-Curtis Index) for 4 samples from each subject during clinical stability, separated by gender. # *p*=NS; * *p* = 0.01, using student unpaired T Test. Error bars represent standard deviation.

In order to assess the differences in bacterial communities between males and females further, the results of bacterial community profiles from the stables samples were analysed using Principle Component Analysis. The results are presented in Figure 5-14 below. There is no significant clustering by gender. This shows that there are no major underlying patterns in the structure of the bacterial community in terms of its composition between males and females. No species show a significant prevalence difference between the genders.

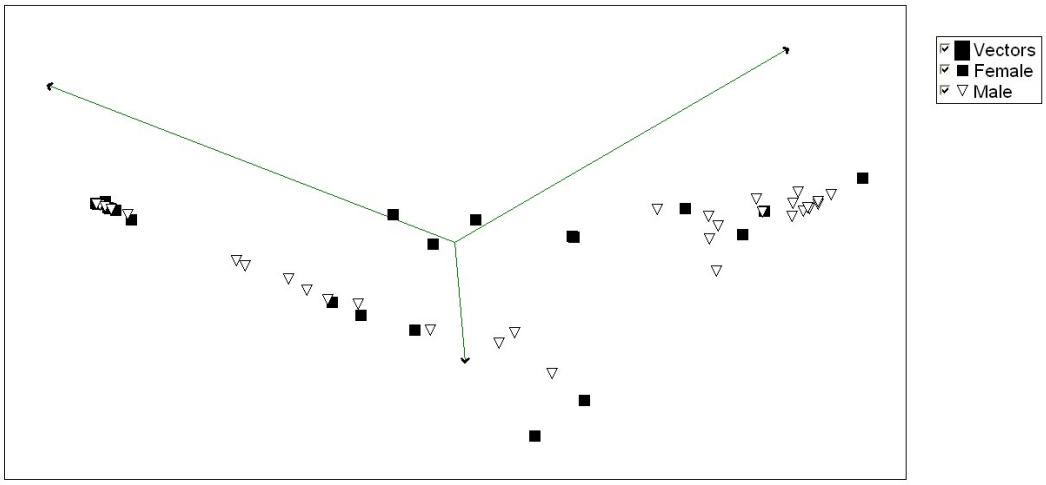


Figure 5-14 Principle Component Analysis of 4 stable samples from each subject, differentiated by gender. Filled-in squares = female; white triangles = males.

5.3 Core/Satellite Analysis of Species Detected

As previously discussed, not all subjects had sufficiently large numbers of samples analysed over the whole study period to allow such analysis. However, Subject 09 had 125 samples analysed, the core-satellite analysis of which are presented in Figure 5-15 below.

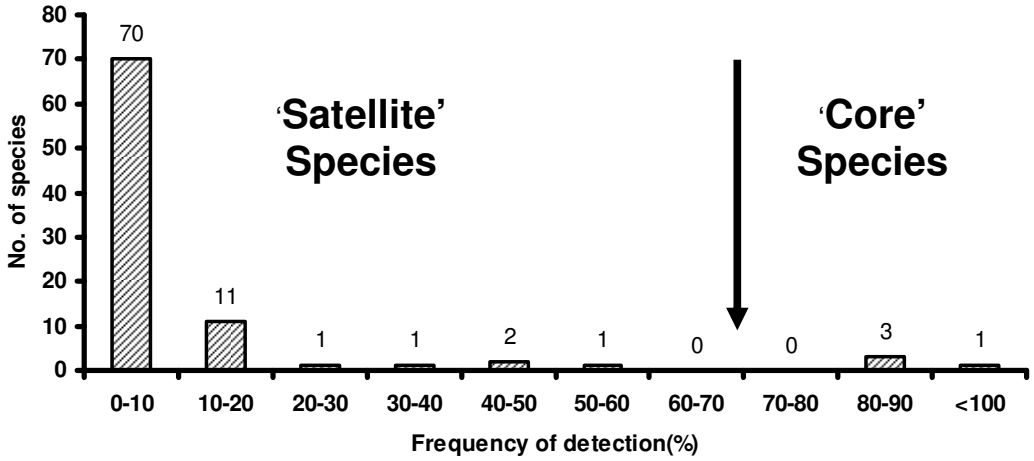


Figure 5-15 Subject 09: Species-Frequency chart to determine constituents of core species (DNA). Numbers above bars represent actual values for each bar. Species to the right of the black arrow (the most frequently detected species) constitute the core species for this subject.

From this, in Figure 5-15 above, it can be seen that a total of four bands constitute the core species, each of which was detected in at least 80% of samples. These can be seen to the right of the dividing black arrow. These four core species of the bacterial community for Subject 09 were:

1. T-RF band length 104 (derived from either *Porphyromonas veroralis* or *melaninogenica*, or *Prevotella pallens*).

2. T-RF band length 634 (derived from *Porphyromonas melaninogenica*).

3. T-RF band length 212: a less specific band length, associated with at least six different species/genera (possible matches include *Neisseria meningitidis, S. maltophilia, N. chinerea, and Actinomyces naeslundii* and *viscosus*)

4. T-RF band length 155: *P. aeruginosa*.

The same analysis was performed on results from RT-T-RFLP, with similar results (data not shown).

5.4 Discussion

Different culture independent techniques were employed to analyse bacterial diversity in CF sputum over a 12 month period. The results from 16S ribosomal RNA gene clone sequence analysis confirm findings from previous studies (Bittar *et al.* 2008; Rogers *et al.* 2005; Sibley *et al.* 2008d) demonstrating higher levels of bacterial diversity than are detected by culture. Interestingly, however, such levels of diversity are in fact much lower than those seen elsewhere. For example, other natural environments may harbour 6000-12,000 different species per gram, depending on the environment (Torsvik, Ovreas, & Thingstad 2002).

The results of the periodic sputum sample analysis by T-RFLP demonstrate two patterns of bacterial community stability over time. Such stability over time has not previously been described in CF respiratory samples. Others in non-clinical studies have however, found similar degrees of stability over time. In bacterial communities from oceanic sediments, Pringault *et al.* (Pringault *et al.* 2007) described quantitative similarity indices over time of over 0.6 (or 60%). This compares to an over all value 0.68 (or 68%) in the subjects of this study. There are a number of subjects who have very stable bacterial communities over time. In these nine “stable” subjects the species hierarchy in the rank abundance curve remaining unchanged in both position and relative proportions. This is despite all but one of them experiencing CFPEs and receiving antibiotics during the study period. Six of this group of eight also received intravenous antibiotics

at least once for a CFPE. In contrast, subjects 4, 5, 9, 10 and, to a lesser extent, 8 and 12, show a high degree of bacterial community instability during the study period. This group included three of the subjects with the worst respiratory function (5, 9 and 12) who had frequent prolonged courses of intravenous antibiotics. However, it also included subjects 8 (who had no CFPEs) and subject 10 who had the least impaired respiratory function of the cohort.

This data can be contrasted to other situations. In an interventional study to assess the effects of 7 days of oral clindamycin on normal faecal gut bacterial communities, Jernberg *et al.* demonstrated large changes at 7 and 21 days after administration of clindamycin, as assessed by Correspondence Analysis (Jernberg *et al.* 2007). These changes persisted in the Bacteroides species population for up to two years, but not for when assessing the overall wider bacterial community. However, more recently Dethlefsen *et al.* reported that, using high a throughput pyrosequencing technique, a short course of oral ciprofloxacin reduced antibiotic diversity for a few weeks only (Dethlefsen *et al.* 2008). By four weeks after treatment bacterial communities structure had recovered to be similar to pre-treatment structure. In a similar finding to Jernberg *et al.,* Dethlefsen *et al.* also noted that some taxa failed to return to pre-treatment levels by 6 months. Thus it appears that the effects of antibiotics on established bacterial communities are short lived, although effects on individual groups of bacteria may be more persistent.

In non-clinical environmental situations, there is little that is comparable to the concept of a CFPE. However, Pringault *et al.* studied microbial communities, using T-RFLP, from tropical marine sediments in summer and winter found no

seasonal fluctuations of bacterial diversity (Bahrani-Mougeot *et al.* 2008; Colombo *et al.* 2009; Tunney *et al.* 2008).

Pseudomonas. aeruginosa was found, by T-RFLP, to be in the top most abundant in 11 of the 14 subjects and was the most consistently dominant species in only 7 of those 11 in which it is found. Thus in 7 of 14 subjects (50%), species other than *P. aeruginosa* were most dominant. None of these other species have been identified as typical CF respiratory pathogens (Lyczak, Cannon, & Pier 2002). The species detected in this study include Species 17 at band length 268, which is otherwise uncharacterised, and was the dominant species in 5 of the 14 subjects. *Prevotella* species were also prominent, being the most dominant species in one subject, and present in the top three species for two other subjects, and a possible candidate in one other. *Prevotella* species are strictly anaerobic (Brenner *et al.* 2005), and frequently found in the gingival crevice in humans, particularly in periodontitis (Aas *et al.* 2005). They are less well characterised as a pathogen beyond the oral cavity, but have been reported in high numbers from the CF lung previously (Tunney *et al.* 2008; Worlitzsch *et al.* 2009) using culture independent and anaerobic culture techniques. Its role as a pathogen in the aetiology of CFPEs and CF lung disease has not been established. Another genus detected that is also a recognised oral inhabitant was *Veillonella* (Bittar *et al.* 2008; Tunney *et al.* 2008). Interestingly, species from this genus have been shown to be capable of forming biofilms and communicating and influencing other bacterial species (Egland, Palmer, Jr., & Kolenbrander 2004). In addition species from the genus *Porphyromonas* also appeared twice in the list of top three species. Species from the genus *Porphyromonas* are also associated with periodontal diseases

(Faveri *et al.* 2009), and have been previously reported from the CF lung (Bittar *et al.* 2008; Tunney *et al.* 2008).

Another frequently detected species was *Rothia dentocariosa*. *R. dentocariosa* is another typical inhabitant of the upper respiratory tract (Aas *et al.* 2005). Species from the genus *Rothia* have been previously detected from airway samples from CF subjects (Bittar *et al.* 2008; Tunney *et al.* 2008). In three subjects it occupied one of the top three positions, each time in the second most dominant position. It is an anaerobe or facultative aerobe (Brenner *et al.* 2005), and has been associated with disease distant to the upper respiratory tract and lung, but only infrequently (Morris *et al.* 2004; Sadhu, Loewenstein, & Klotz 2005).

It is note-worthy that the two subjects that appeared to have the greatest bacterial species "instability" over time were subjects 05 and 09. They were the most frequent exacerbators, had the worst lung function, and both have since gone on to be assessed for possible lung transplantation. It not possible to determine cause and effect, but there are a number of possible explanations for this link between pulmonary disease severity and bacterial community instability. First, it is possible that the frequent courses of intravenous antibiotics (5 and 6 courses of IV antibiotics respectively) that these two individuals received drove the flux in bacterial species seen. Although plausible, there is little evidence from the work presented here, or other published work (Dethlefsen *et al.* 2008; Jernberg *et al.* 2007) that IV antibiotics (and other associated treatments) do in fact have any dramatic long term effect on bacterial communities. A second possible explanation is that such flux is in fact the driving factor behind the worse pulmonary health. This may be a partial

explanation as there is a correlation between lung function and structure of the bacterial community (see Figure 5-11 above). Additionally there may be a relationship between lower lung function and less similarity between adjacent samples using the quantitative Bray-Curtis Index (see Figure 5-10). There is a further explanation based on heterogeneous lung damage and mucociliary clearance. The CF lung becomes damaged in a heterogeneous way: some parts of the lung are more damaged than others. With the heterogeneity comes the possibility that separated, or partially separated communities exist with a given set of lungs. These separate environments may develop due to different amounts of ventilation (Gustafsson *et al.* 2008) and perfusion that may produce different habitats. In addition stochastic processes are known to be important in the composition of bacterial communities (van der Gast, Ager, & Lilley 2008). This is likely to lead to different communities of bacterial in different parts of the damaged lung. In addition, as more areas of the lung become less well ventilated, inhaled antimicrobial therapies will not reach the worst affected parts, particularly those areas distal to impacted inspissated secretions. As lung function worsens this increased heterogeneity of lung habitats may result in sputum samples containing a more varied composition of bacteria, from different parts of the damaged lung. Support for a heterogeneous distribution of inflammation during CF pulmonary exacerbations comes from studies using recent advances in imaging (Klein *et al.* 2009), as well bronchoscopic studies assessing inflammation and microbiology from different lobes (Gutierrez *et al.* 2001) Additional support for a theory of regional bacterial heterogeneity comes from studies of serial induced sputum in CF. Rogers *et al* showed that in any one given induced sputum sample an average of 58% of the total expectorated

bacterial community, as detected by T-RFLP, could be detected (Rogers *et al.* 2010).

The significance of bacterial community stability over time is unclear. The first point is that it is surprising that the bacterial communities are so persistent over time. Clinicians and patients make vigorous, expensive and time-consuming attempts to treat, disrupt and challenge the bacteria of the lower airway in CF (Elkins *et al.* 2006; Fuchs *et al.* 1994; Ramsey *et al.* 1999). Despite this effort, relatively little impact appears to be made on these bacterial communities of the lower airways during the course of one year. However, it should be made clear that all subjects were on long term antimicrobial therapy, and thus it is possible that treatment is contributing to the stability of the bacterial community, perhaps by preventing the establishment of new species in the pre-existing bacterial community.

The differences in bacterial communities seen between the genders are of interest. The evenness, or slope, of the bacterial community was found to be statistically steeper in females than males (see Section 5.2.4 above). There is no evidence to support an explanation for such a finding, but it is interest that females with CF are reported to have a faster rate of decline in lung function than males, and thus a shorter overall life expectancy (Corey *et al.* 1997; Liou *et al.* 2001). In addition there are significant differences in similarity over time between those with a more severe genotype, and those with a milder genotype. Those with a milder genotype maintain greater similarity over time than do those with a more severe genotype (Corey *et al.* 1997; McKone, Goss, & Aitken 2006). Given that those with a more severe genotype have more severe pulmonary disease and shorter life expectancy, this could suggest that a more

stable community is associated with less severe disease. However, those with more severe disease may also receive more antimicrobial therapy.

The presence of large quantities of anaerobic bacteria identified by clone sequence analysis and also detected longitudinally by T-RFLP is unsurprising when reviewing recent literature. Many other researchers have produced similar findings. It is noteworthy that wide range of techniques for detection have been employed, including culture of at least some samples obtained bronchoscopically from the lower respiratory tract secretions (Harris *et al.* 2007; Tunney *et al.* 2008). This point is of importance because the oropharynx is well known to be an environment that harbours many species of anaerobic bacteria, and samples obtained by passage of secretions directly through the oropharynx, as is the case with spontaneously expectorated samples, have the theoretical potential to be contaminated with these bacteria. Samples obtained bronchoscopically avoid this potential, and thus this work, along with work comparing sputum and mouthwash samples (Tunney *et al.* 2008) demonstrates that anaerobic bacteria in sputum samples really are from the lower respiratory tract and not contamination from the oropharynx.

Once this point is accepted, that anaerobic bacteria really are present in the lower airways of patients with CF, the question arises as to where they originated from. Answers to such questions might provide opportunities to intervene therapeutically to prevent, delay or reduce the immigration of bacteria to the lower airways, and potentially reduce bacterial induced lung damage.

Chapter 6 - Results: Flux around treated CF Pulmonary Exacerbations

Due to the very large number of samples collected in the course of this study, and the involved and labour intensive process by which samples were analysed by T-RFLP and RT-T-RFLP, it was not possible to analyse all samples received. For patients experiencing a CFPE, one example from each was studied. At least three samples prior to each CFPE, and from at least 21 days before the start of systemic treatment, were analysed. At least three samples from during the treatment period were analysed. If possible, one or more samples were analysed from after the end of systemic antibiotics. The analysis of samples starting 21 days before the treatment period was chosen as a clinical and microbiological decision based on experience of the time course in fluctuations in symptoms. Additional time points were chosen in an attempt to capture a range of variations across the whole course of a CFPE, before, during and after treatment. The antibiotics used for each of the analysed exacerbations are presented in Table 6-1 below. When a subject had more than one CFPE, the decision as to which one to analyse was taken on the basis that the driving forces for change leading to a CFPE would be the same, irrespective of the type of treatment given for that CFPE (*i.e.* oral or intravenous antibiotics).

Subject Number	Antibiotic One	Antibiotic Two	Antibiotic Three
1	Ciprofloxacin PO		
2	Colomycin IV	Tobramycin IV	
4	Doxycycline		
5	Meropenem IV	Amikacin IV	Ciprofloxacin PO
6	Ceftazidime IV	Gentamycin IV	
7	Doxycycline		
9	Meropenem IV	Colomycin IV	
10	Ceftazidime IV	Gentamycin IV	
11	Clarithromycin		
12	Ciprofloxacin PO	Metronidazole PO	
13	Ceftazidime IV	Gentamycin IV	
14	Meropenem IV	Tobramycin IV	

Table 6-1 Antibiotics used in each of the CFPEs analysed in chapter 6.

In addition to the analysis of one CFPE from each subject, three subjects were selected to have all of their CFPEs analysed. These three subjects were representative of three different clinical phenotypes: subject 02, subject 09 and subject 14.

There are other chronic respiratory conditions such as COPD in which bacteria persist in the lower airways. In COPD Patel *et al.* demonstrated that it is useful and valid to divide patients into frequent exacerbators and infrequent exacerbators (Patel *et al.* 2002). Such an approach was adopted here to determine if any bacterial community characteristics pre-disposed to more frequent CFPEs. In the work of Patel *et al.*, the division between frequent vs. infrequent exacerbations was determined to be 2.58. For the practicality necessities of this study, subjects were defined as Frequent Exacerbators if they had $\geq$3CFPEs per annum (in the study period), and infrequent if they had less than three CFPEs. See Table 6-2 below.

	Frequent Exacerbator	Infrequent Exacerbator
Subject No.	1, 2, 4, 5, 7, 9, 11, 12, 13, 14.	3, 6, 8, 10

Table 6-2 Classification of Subjects by Frequent vs. Infrequent Exacerbators

6.1 Variations in Species Richness

6.1.1 Single CFPE from Each Subject

Species richness varied over time. Time Point Zero is for samples taken immediately before the commencement of therapy for a CFPE. At 21 days before the start of treatment, mean (±SD) bacterial species richness was 6.67 (±3.25) per sample, compared to 4.42 (±2.60) per sample at day zero (p= 0.04 by paired t test). Figure 6-1 below shows these variations. There is no clear recovery in species number either during or after treatment.

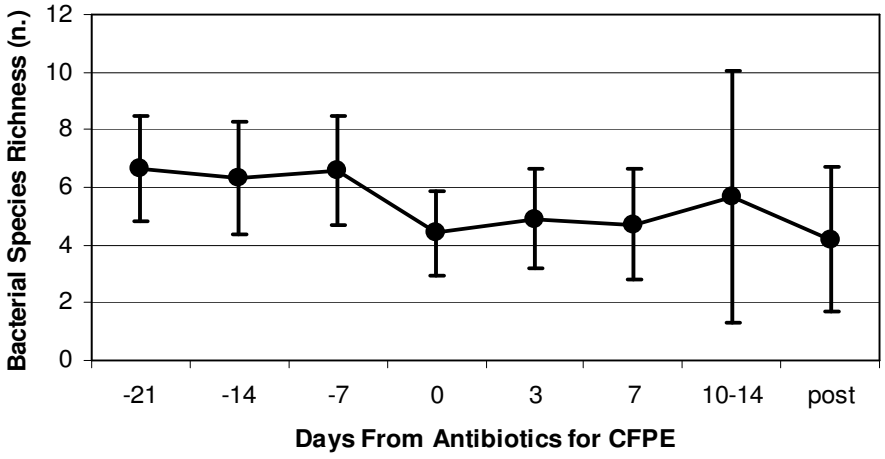


Figure 6-1. Bacterial species richness before, during and after antibiotic treatment for CFPE.

	Mean species number (±S.D.)	**Comparison of mean to day zero; *p* value**
Day -21	6.67 (±3.25)	0.04*
Day -14	6.34 (±3.47)	0.03*
Day -7	6.58 (±3.34)	0.08
Day Zero	4.42(±2.60)	N.A.

Table 6-3 Comparison of mean species richness between time points before CFPE, using paired t test. *= statistically significant at 0.05 level.

Species richness at Day-14 before the start of a CFPE was 6.3 (±3.5) per sample (p=0.03 compared to Day-0; paired t test), and at Day-7 was 6.6 (±3.3) per sample (p=0.08 compared to Day-0; paired t test).

Four periods were grouped (Pre-CFPE; day zero (pre-antibiotics); during antibiotics; and Post-antibiotics), the mean number of species detected per sample were as follows 5.9 (n=36; SD 3.4), 3.6 (n=12; SD=2.2), 4.8 (n=23; SD 3.1). Mean bacterial species richness was statistically higher in the pre-CFPE period than at the time of CFPE (p=0.03). No other significant differences between the periods in relation to bacterial species richness were observed. See Figure 6-2 below.

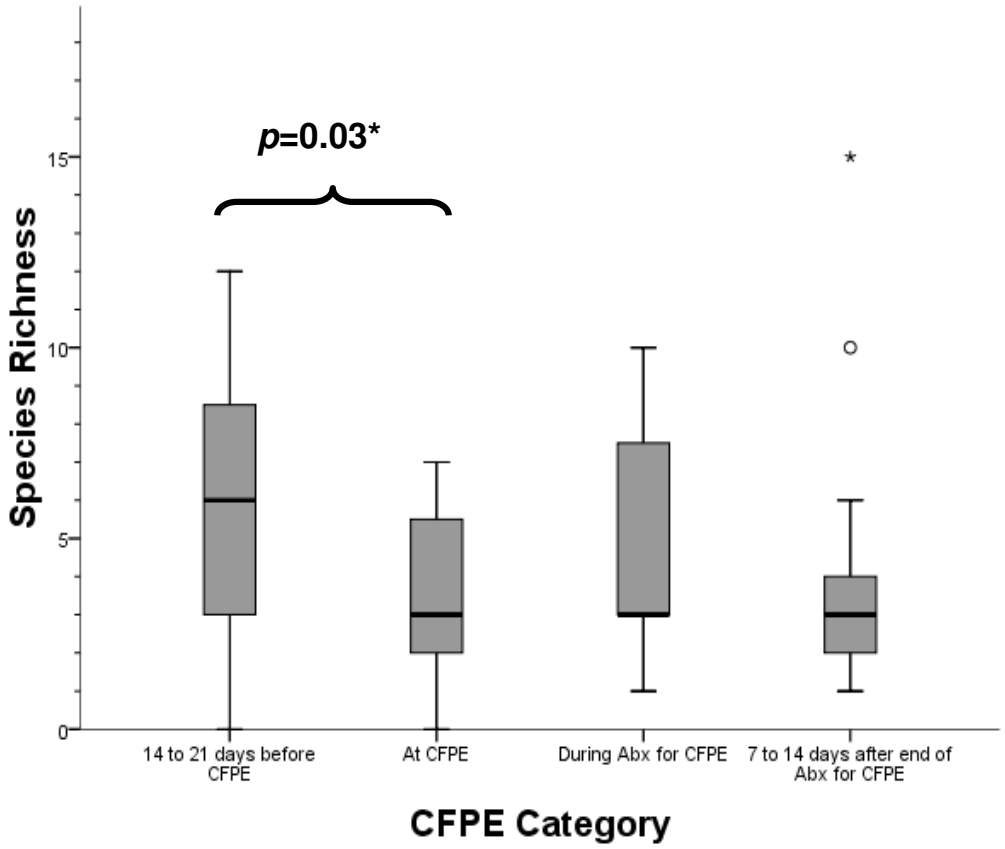


Figure 6-2 Species Richness before during and after antibiotic treatment for CFPE. * = p value significant at 0.05 value. All other pairings p=N.S., using unpaired t test.

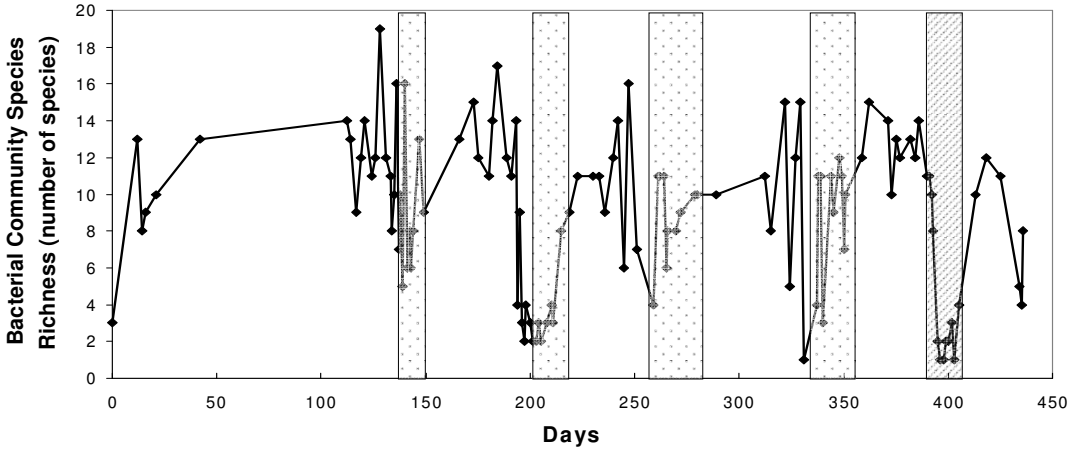


Figure 6-3 Bacterial Community Richness for Subject 09. Results are from DNA profiles not treated with PMA. Boxed areas denote systemic antibiotics, the first four ('stippled') areas were clinician defined CFPEs, whilst the fifth ('hatched') area denotes elective antibiotics.

In order to ascertain if similar patterns of perturbations in the bacterial community to those seen in one CFPE from 12 different subjects would also occur in CFPEs from just one subject, all the analysable CFPEs from subject 09 were analysed (CFPE n=4). Species richness in the three periods before during and after treatment for a CFPE, mean (±SD) species richness was 10.2 (±4.5), 7.5 (±3.8), and 11.6 (±2.2) species respectively. Using the student's unpaired t test, the probability of the differences between groups being significant was: Pre *vs.* during, p= 0.007; pre *vs.* post p=ns; during *vs.* post $p<0.0001$. This suggests that the number of species detected before antibiotic and after antibiotics are given is much higher than during a period of systemic antibiotics.

6.2 Variations in Species Evenness

In order to assess the evenness of the bacterial community, the slope of the logged values of the ranked abundances was calculated (see 2.6 above). Mean values for at various time points before, during and after the start of treatment for a CFPE, are presented in Figure 6-4 below, with a mean FEV1 plotted for comparison.

In Figure 6-4 a trend towards a steeper slope in the bacterial community at the time of CFPE compared to the preceding 21 days was observed. This was not statistically significant with $p>0.05$ in all comparisons.

In the time periods both during and following the administration of antibiotics, no clear trend was observed. However, it is worth noting that the introduction of treatment for a CFPE has not resulted in the return of community evenness to pre-CFPE levels.

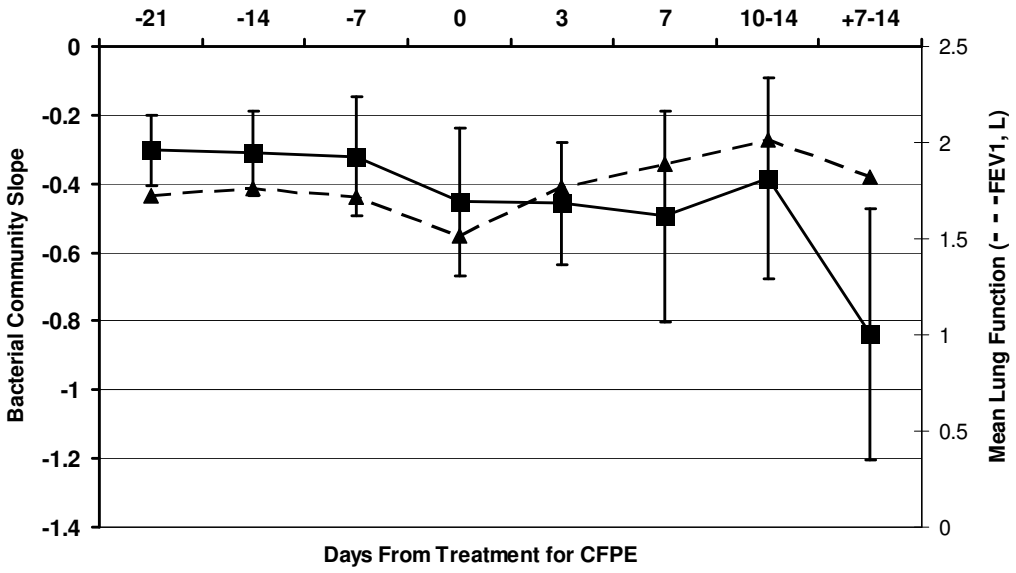


Figure 6-4. Changes in Bacterial Community Slope Before, During and After Treatment for a CFPE. Error bars represent 95% Confidence Intervals. --▲--= mean FEV1 for same time period. All samples treated with PMA.

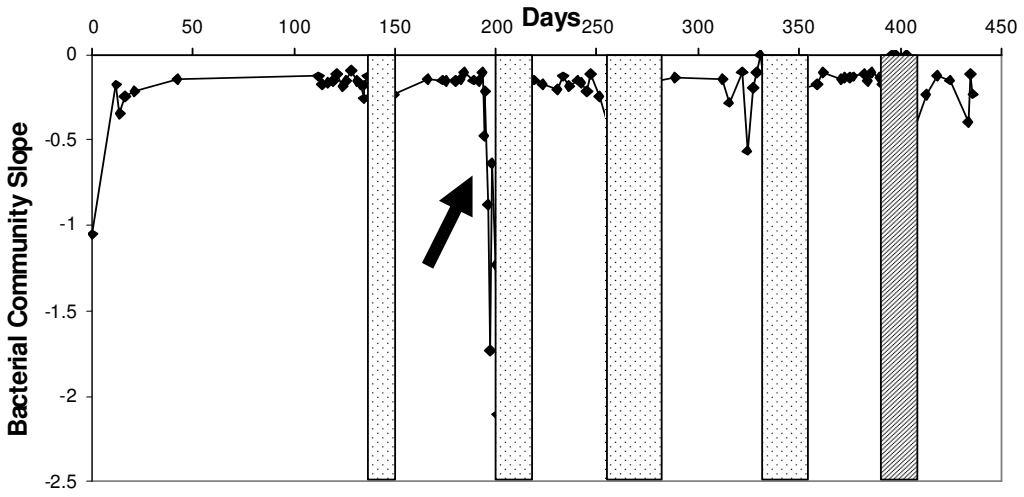


Figure 6-5 Changes in bacterial community slope for Subject 09. Stippled areas denote treatment periods for CFPEs; hatched area denotes 'elective' treatment including antibiotics. Results are from DNA profiles (without PMA treatment). Black arrow – see text.

Subject 09

In order to ascertain if similar perturbations in bacterial community slope, seen in one CFPE from 12 different subjects, would also occur in CFPEs from just one subject, all the CFPEs from subject 09 were analysed (CFPE n=4). Results are presented in Figure 6-5 above. It was seen that bacterial community slope in the three periods before during and after treatment for a CFPE, mean slope of the bacterial community (±SD) was -0.28 (± 0.31), -0.54 (± 0.56), and -0.16 (± 0.02) respectively. Using the student's unpaired t test, the probability of the differences between groups being significant was: Pre *vs.* during, p= 0.02; pre *vs.* post p= 0.02; during *vs.* post p= 0.0004. It can also be seen that between each course of antibiotics, the Slope is consistently around a value of approximately -0.2.. Other patterns are less consistent: In some CFPEs the slope becomes noticeably less even prior to the start of treatment (above: 2nd CFPE, marked with black arrow), whereas overall the mean slope in the three weeks prior to the start of treatment is more even than that during treatment.

6.3 Variations in Flux between Samples

It was hypothesised that there may be greater flux in bacterial species and within the bacterial community around the time of a CFPE. In Figure 6-6 below is shown the results of Sorensen Similarity Index for the periods of time before a CFPE (days -7 to -21), for the day of CFPE compared to the pre-CFPE period, and for the period during treatment. Mean (±SD) Sorensen Index for the three time periods were 0.63 (±0.20), 0.51 (±0.25), and 0.48 (±0.29). All differences were not statistically significant.

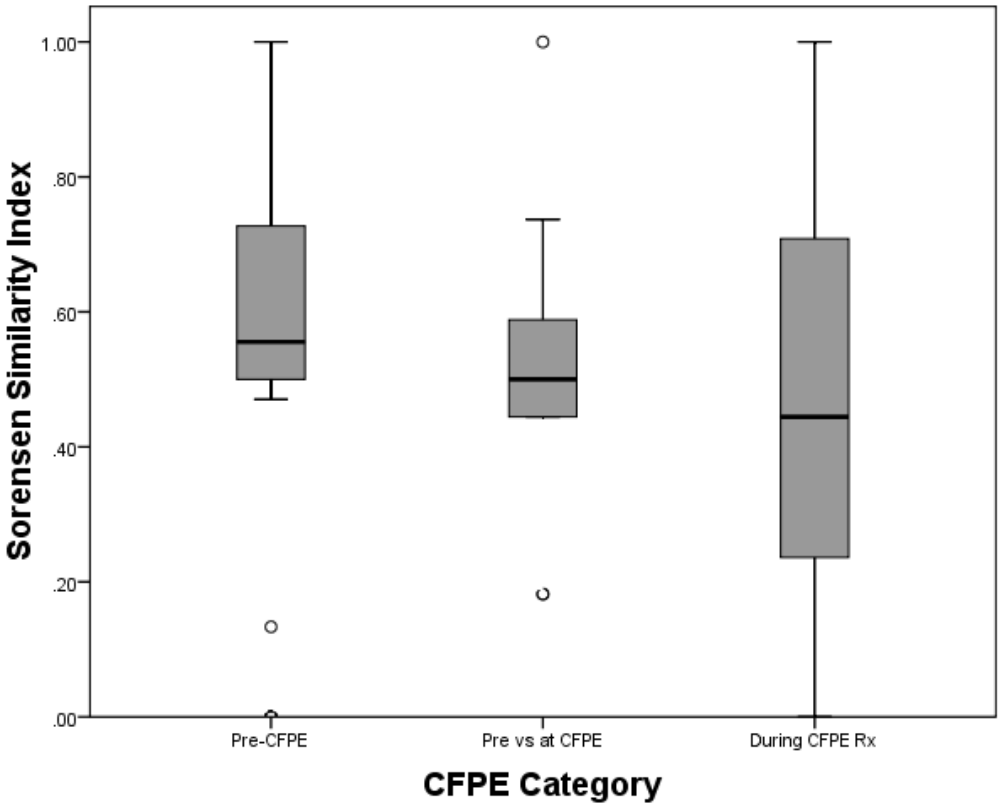


Figure 6-6 Box plot of Sorensen Similarity Index around time of CFPE.

6.4 Variations in Key Species

Pseudomonas aeruginosa has been considered a key pathogen in CF (Lyczak, Cannon, & Pier 2002). The changes in the proportions of *P. aeruginosa* present in sputum samples taken before during and after CFPE treatment for 12 CFPEs is presented below inFigure 6-8. It can be seen that proportions of *P. aeruginosa* are relatively low in the time preceding a CFPE (mean 42±5.6%), and rise during (mean 58.6±7.8%) and after treatment (mean 68.1±10.9%). These differences were not statistically significant. This is also presented in Figure 6-7 below.

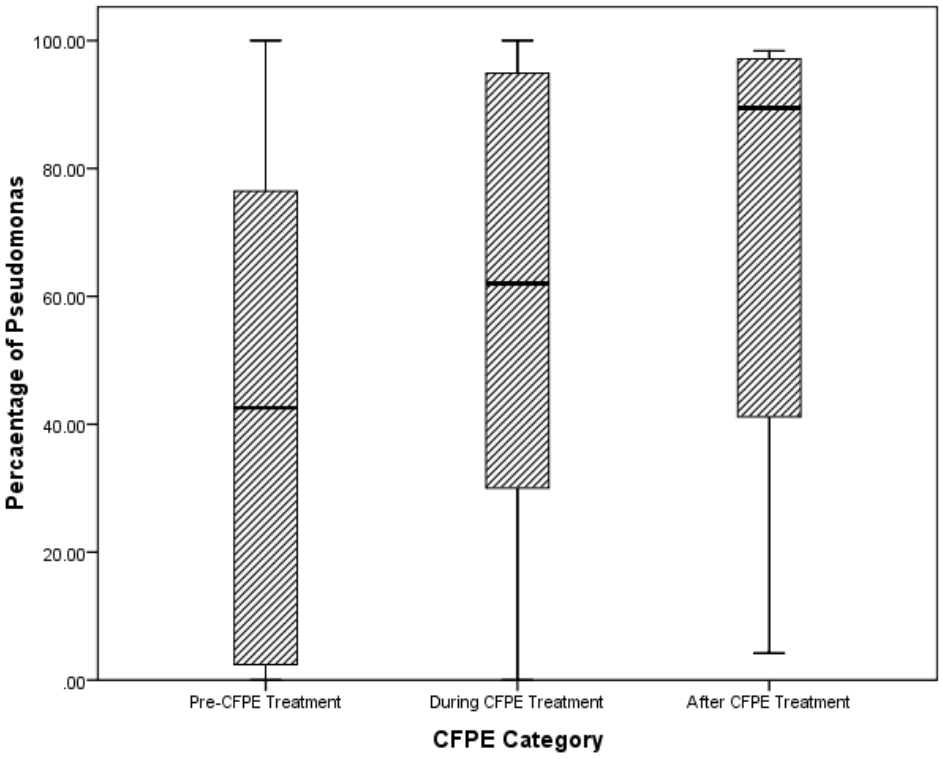


Figure 6-7 Box plot of proportions of *P. aeruginosa* around CFPE. Between groups difference *p*= ns (one way ANOVA).

It can be seen that, in contrast to *P. aeruginosa*, levels of *Prevotella* are higher before treatment for a CFPE, decline rapidly on treatment, and remain low after treatment. The differences between the three time categories was found to be highly significant by one way ANOVA (p=0.0002).

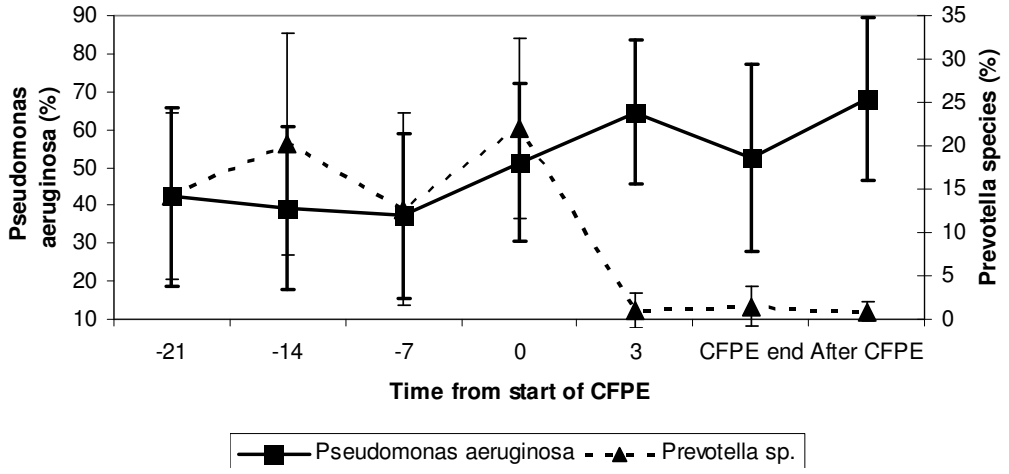


Figure 6-7 Fluctuations in proportion of *Pseudomonas aeruginosa* and *Prevotella* species present before during and after CFPE treatment. Error bars represent 95% confidence intervals

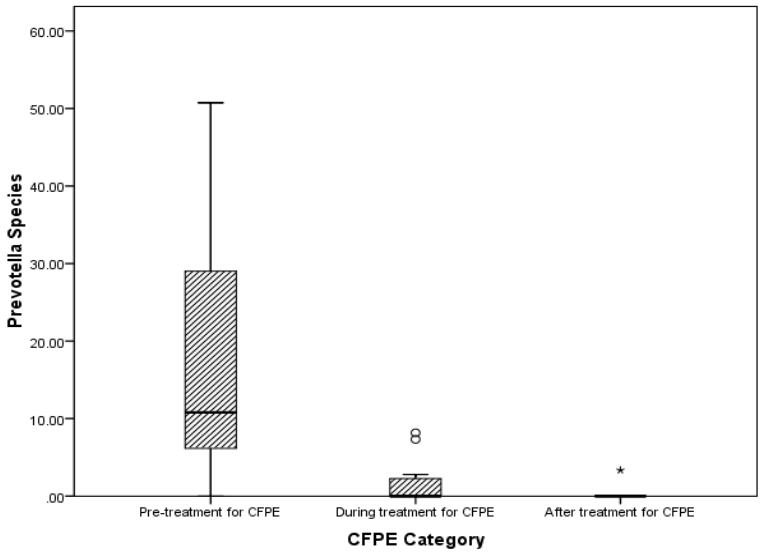


Figure 6-8 Box plot of proportions of *Prevotella* spp. around CFPE. Between groups difference p=0.0002 (one way ANOVA).

Prevotella spp. were not the only obligate anaerobes detected. In Figure 6-9 below, bands that are associated with species that are known to be obligate anaerobes (Brenner *et al.* 2005) are plotted against changes in *P. aeruginosa*. A strict definition of which bands were from obligate anaerobes was applied. Thus there were other band lengths that had obligate anaerobes associated

with them, but not exclusively so. These band lengths also had other species not considered obligate anaerobes associated with them and were thus excluded from this definition of bands derived from obligate anaerobes. This strict definition of which data to include in this analysis may well have resulted in the proportions of obligate anaerobes being an underestimation of the true proportions of anaerobes. The proportion of anaerobes fall from a mean of 25% (±20.4) on the day prior to CFPE treatment, to 6.35% (±7.7) at the end of antibiotics (p= 0.03 by paired t test). This difference persists after the end of the treatment.

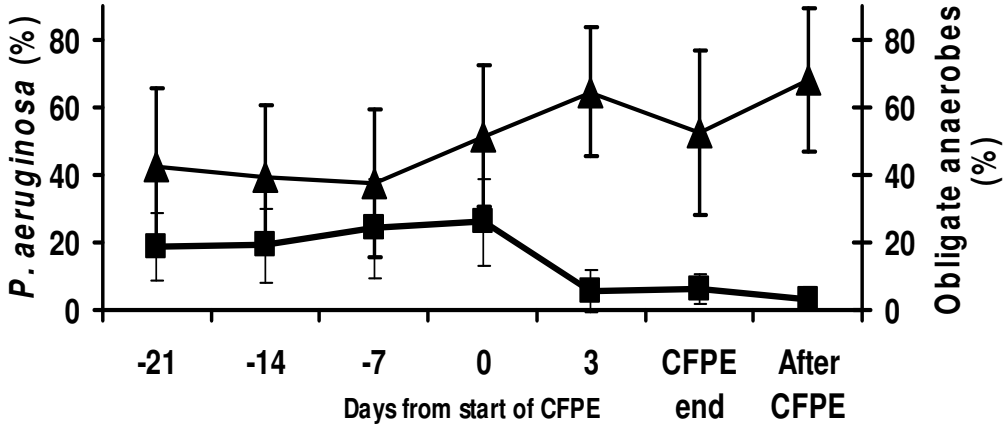


Figure 6-9 Proportions of obligate anaerobes and *P. aeruginosa* around CFPE. ▲ = *P. aeruginosa*. ■ = Anaerobe. Error bars represent SD.

Subject 09

The effect of antimicrobial therapy on *Prevotella* spp. proportions throughout a whole year, including CFPEs is shown in Figure 6-10 below. It can be seen that reduced relative abundance of *Prevotella* species has a temporal relationship with the periods of systemic antimicrobial therapy, particularly for the 2nd, 3rd and 4th episodes. During the first CFPE in was noted that there no noticeable

effect of systemic antibiotics on the proportions of *Prevotella* spp.. The first CFPE was the only one in which the intravenous antibiotic Meropenem was not a part.

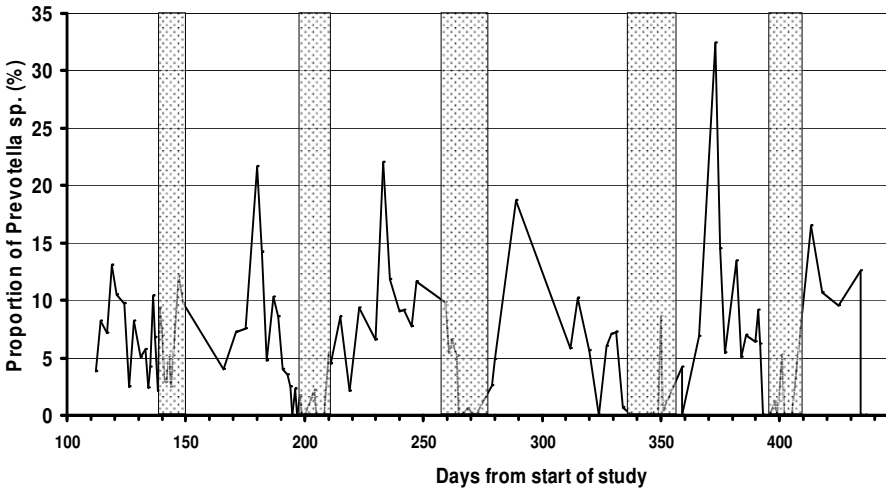


Figure 6-10 Effects of periodic antimicrobial therapy on relative abundance of *Prevotella* species for one Subject (09). Grey areas represent periods of systemic antimicrobial therapy.

T-RF Band 268, identified by PCA as an important species in the differentiation between frequent and infrequent exacerbators in samples analysed as part of the stability analysis. In this analysis of samples around the time of an exacerbation, Band 268 was almost never detected (only identified in Subject 12).

6.5 Multivariate Analysis

Using results from samples taken from subjects when clinically stable (see Chapter 5 -), but dividing subjects into Frequent and Infrequent Exacerbators,

as per Table 6-2 above, it was possible to analyse bacterial community similarities in samples using Principle Components Analysis

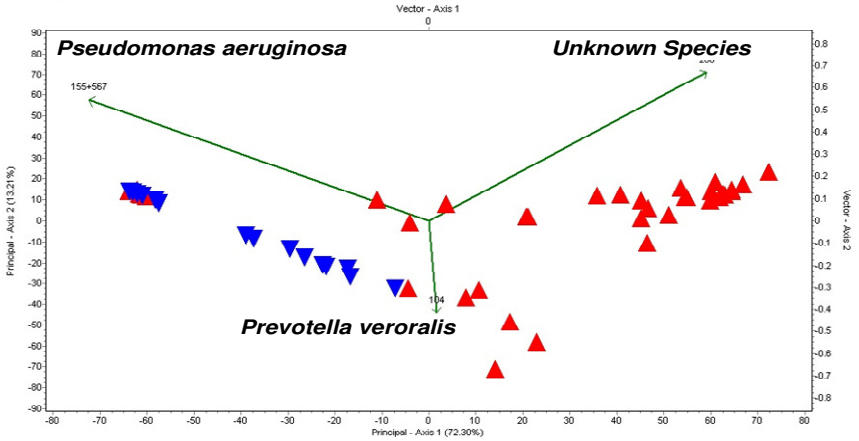


Figure 6-13 Principle Component Analysis of samples from subjects when stable, divided in to infrequent CFPEs ('point down' triangles) and frequent CFPEs ('point up' triangles).

In Figure 6-13 above, it can be seen that samples from infrequent exacerbators (blue triangles) cluster together, where as the frequent exacerbators (red triangles) are more widely distributed. The two Principle Components responsible for this clustering are an unknown species (T-RF 268), and *P. aeruginosa*. Finally it is of note that a third component, band 104 – a *Prevotella* species, is also a Principle Component of the differentiation between samples. On further statistical testing using Wilcoxon rank sum test, the median proportions of *P. aeruginosa*, for 'frequent exacerbators' and 'infrequent exacerbators' respectively were: 6.6% vs. 12.1% (p= 0.187); *Prevotella* spp. were 0.0% vs. 0.30% (p= 0.006), and T-RF 268 4.5% vs. 0.0% (p<0.001)

	Frequent Exacerbators	Infrequent Exacerbators	*p* value
T-RF 155 (*P. aeruginosa*)	Median 6.6% (IQR 70.9%)	12.1% (38.5%)	0.187
T-RF 104 (*Prevotella* spp.)	Median 0.0% (IQR 0.0%)	0.30% (4%)	0.006*
T-RF 268	Median 4.5% IQR 8.6%)	0.0% 0.0%	< 0.0001*

Table 6-4 Comparison of median values for bands identified as principle components by PCA. Statistical analysis by Wilcoxon rank sum test. * = statistically significant result.

6.6 Discussion

A number of pertinent findings emerge from these data, relating primarily to two key areas, namely to bacterial community structure, and key species.

6.6.1 Bacterial community structure

Of the twelve subjects who experienced a CFPE during the study period, a clear effect of treatment on bacterial community structure can be seen. Species richness falls significantly during treatment. This is unexpected and not in keeping with studies of the effects of antibiotics on other bacterial communities. Robinson *et al.* assessed the effects of antibiotics on bacterial communities using a butterfly larval model and noted marked increases in bacterial species detected during treatment (Robinson *et al.* 2009). Bacterial community slope becomes significantly steeper during treatment compared to both the immediate pre-treatment period, and in the case of Subject 09, compared to the longer term baseline too. However the changes in values of both bacterial species richness and community evenness during treatment return to pre-treatment

values. Given that treatment ends due to a symptomatic improvement, which persists, it is a little perplexing that the apparent effects of treatment, including intensive antibacterial treatment, are so quick to dissipate. However, in all cases, some form of less intensive long term antimicrobial therapy continued. It has also been shown that bacterial community evenness offers advantages to the persistence of the bacterial community under pressure in a theoretical model (Wittebolle *et al.* 2009).

It was found that, during treatment, both the number of species detected and the evenness of the bacteria fall. This is to be expected. For example, as a community becomes more dominated by a single species, giving a steeper slope, many of the rarer species may fall below the lower limit of detection, resulting in a fall in the number of species detected (Ager *et al.* 2009).

The fall in species richness and increase in the steepness of the slope during treatment for CFPEs is at least in part due to a rise in the relative abundance of *P. aeruginosa* and fall in the relative abundance of other species, notably *Prevotella spp.* (see Figure 6-7). *Prevotella spp.* have been previously detected and identified by both culture-based (Tunney *et al.* 2008) and sequence based, culture-independent techniques (Bittar *et al.* 2008). Tunney *et al.* found *Prevotella spp.* as the most frequent detected anaerobe in samples obtained by bronchoscopic lavage and spontaneous sputum from adult CF subjects (Tunney *et al.* 2008). Harris *et al.* also found a number of *Prevotella* species in CF subjects , but from a younger population with a mean age of 9.1 years (Harris *et al.* 2007). Both of the afore-mentioned studies were horizontal descriptive studies, with no evidence to support the importance or otherwise of the diversity of species detected.

The finding that treatment for CFPEs, of which intensive antimicrobial therapy is the mainstay, results in a *rise* in the relative quantity of *P. aeruginosa* compared to other species runs counter to the prevailing understanding of CFPE treatment. Gibson *et al.* reviewed the management of CFPEs in 2003, highlighting that the only strategy for selection of antibiotics was based on the presence and resistance patterns of *P. aeruginosa* (Gibson, Burns, & Ramsey 2003). As previously discussed in the Introduction(see Section 1.1) others have highlighted both the lack of correlation of these resistance patterns to clinical outcomes (Smith *et al.* 2003), and the lack of internal consistency of the result from antibiotic resistance pattern results to *P. aeruginosa* (Foweraker *et al.* 2005; Foweraker *et al.* 2009). However, it should be stressed that the results presented in this work only show a relative increase in *P. aeruginosa* levels and that it entirely possible, and indeed clinically likely, that absolute quantity of *P. aeruginosa* fall during CFPE treatment (Regelmann *et al.* 1990). Were it to be demonstrated that absolute *P. aeruginosa* levels fall, this work demonstrates that the effects of treatment on other species than on *P. aeruginosa*.

The results of Principle Component Analysis and the resulting comparison of means of the principal components identified is important. As can be seen from Table 6-4 above, there were significant differences between species in the frequent exacerbators vs. infrequent exacerbators. There was significantly more *Prevotella* spp. and significantly less (=none) of T-RF 268 in the infrequent exacerbators. There was also a non-significant trend towards more *P. aeruginosa* in the infrequent exacerbators. The finding of key species that contribute to exacerbation frequency would of of importance in identifying

mechanisms behind CFPEs, which are such significant events for people with CF.

Another group of bacteria that have been implicated in the development of CFPEs has been the Streptococcus milleri group (SMG). This work has found no evidence to lend support to the hypothesis that bacteria from the SMG play any significant role in CFPEs. However, in work done within this research group, samples from a CF centre in the USA were compared to samples from CF patients in Southampton. Samples from the two groups were significantly different to each other and clustered geographically (unpublished data). This might offer an explanation as to why bacteria from SMG were found in one centre but not another.

Chapter 7 - Inflammatory Mediators

Although it is certainly true that the presence of large quantities of bacteria in the lower airways of patients with CF is an important driver in the pathological destruction of the lung parenchyma that leads to morbidity and ultimately death, the link between fluctuations in the structure of that bacterial community and the aforementioned lung damage is not clear. In order to link measures of bacterial community structure, and the fluctuations therein, to the response of the host to their presence, sputum inflammatory mediators were measured as outlined in Section 2.4 above. As the sample handling requirements were more exacting for samples for inflammatory mediator measurements (Ordonez *et al.* 2003) compared to samples for microbiology analysis (Mayer-Hamblett *et al.* 2007), a separate sample set was collected which was delivered straight to a -80^{o}C freezer within two hours of expectoration. In total, 70 analysed samples were collected from the fourteen subjects. A number of samples that were collected were subsequently excluded from parts of the analysis as they were not obtained at times that were easily differentiated in to 'CFPE (pre-treatment)', and stable. For the purposes of the study, stable was defined as at least three weeks before or after a CFPE.

A summary of the results are presented in Table 7-1 below. In general there was wide variability in results both within and between subjects. There were also generally low numbers of samples for each subject and category (stable/CFPE). From Table 7-1 it can be seen that there were only three occasions in which a subject had more than two samples in both stable and CFPE in any given inflammatory mediator (Subject 09: NE; Subject 12: NE and ECP). In addition, paired samples were not obtained. In other words, samples were not obtained

before and after a given CFPE, but instead before one CFPE but perhaps after an additional CFPE. This was due to the difficulty in obtaining appropriate samples, given the more exacting sample handling requirements, in what was essentially a community based study.

During the measurement of the inflammatory mediators in the soluble phase of the sputum supernatant, it was necessary to estimate the expected range for results in order to allow the result to fall within the standard curve. Unfortunately, on a number of occasions values were much higher than had been predicted and fell above the standard curve, preventing acceptably accurate measurements. These results were excluded. This occurred for four results for IL-8.

From Table 7-1 it can be seen that the median value for all three measured inflammatory mediators was higher during a CFPE than at times of clinical stability. However, it can be seen that none of the differences reached statistical significance (analysed using non-parametric tests due to the non-parametric distribution of the data).

	Stable			Exacerbation		
	N.E. (mu/μL)	**IL-8** (ng/ μL)	**ECP** (ng/ μL)	**N.E.** (mu/μL)	**IL-8** (ng/ μL)	**ECP** (ng/ μL)
1. mean	1.51	25.2	1.93	2.04	101.3	10.2
(N)	(2)	(2)	(2)	(2)	(2)	(2)
2. mean	5.86	40.8	5.61	3.36	47.1	9.08
(N)	(3)	(3)	(2)	(2)	(1)	(2)
3. mean	3.11	62.4	3.08	N/A	N/A	N/A
(N)	(6)	(4)	(4)	(0)	(0)	(0)
4. mean	2.73	92.2	N/A	0.441	11	0.12
(N)	(1)	(1)	(0)	(1)	(1)	(1)
5. mean	3.01	41.5	N/A	1.64	33.6	3.01
(N)	(1)	(1)	(0)	(2)	(1)	(2)
6. mean	2.97	19.80	5.49	2.56	54.30	7.07
(N)	(3)	(2)	(1)	(2)	(2)	(2)
7. mean	7.61	63.70	1.96	4.10	77.90	6.29
(N)	(1)	(2)	(1)	(3)	(2)	(2)
8. mean	1.78	34.50	4.88	N/A	N/A	N/A
(N)	(3)	(3)	(1)	(0)	(0)	(0)
9. mean	14.30	89.90	13.19	20.32	209.65	12.82
(N)	(3)	(2)	(2)	(7)	(7)	(5)
10. mean	9.77	3.67	13.207	0.424	35.2	5.37
(N)	(1)	(1)	(1)	(1)	(1)	(1)
11. mean	2.46	56.55	12.12	0.98	N/A	1.93
(N)	(2)	(2)	(2)	(1)	(0)	(1)
12. mean	3.30	52.88	3.03	3.14	64.05	4.89
(N)	(6)	(5)	(6)	(3)	(2)	(3)
13. mean	1.23	53.80	6.30	3.20	50.50	6.83
(N)	(1)	(2)	(2)	(3)	(2)	(3)
14. mean	0.54	21.15	2.11	1.23	39.23	2.77
(N)	(2)	(2)	(2)	(3)	(3)	(3)
Overall Median	2.71 p=0.071	43.60 p=0.211	3.81 p=0.101	2.80	56.90	5.65

Table 7-1 Inflammatory Mediator results for samples taken during Pulmonary Exacerbations and in periods of clinical stability. Difference between mean values for each mediator at the two time points assessed using Wilcoxon signed rank test.

7.1.1 Inflammatory Mediators *vs.* Bacterial Community Measures

If fluctuations in the structure of the complex bacterial community present in CF sputum were to have some bearing on the well being of the host, then it might be expected that changes in sputum inflammation might correlate with measures of community structure. In Table 7-2 (below) are presented correlations between a number of different measures of bacterial community structure and the inflammatory mediators measured from the same samples. For more details see Section 2.5 above.

The measures of species evenness used were the slope of the log values of the ranked species dominance plot, in addition to the percentage abundance of the most dominant species in each sample, and the percentage abundance of *P. aeruginosa* in each sample. The measure of bacterial community richness was number of species. These measures were performed for both T-RFLP results (bacterial DNA) and RT-T-RFLP results (bacterial RNA).

Values presented are for all samples from all subjects, irrespective of whether samples could be clearly delineated into or out with periods of CFPE. Values were not normally distributed, and thus non-parametric Spearman's rank correlation was used. When assessing correlations between DNA (T-RFLP) and inflammatory mediator results there were no statistically significant correlations between measures community structure, either evenness or richness, and measured sputum inflammatory mediators. However, when looking at RNA results (RT-T-RFLP), which theoretically is a marker of bacterial activity rather than merely bacterial presence or absence, there were two

correlations that were statistically significant which are described in more detail below. Values of Spearman's rho were not large (max value -0.346).

For RNA (RT-T-RFLP) results there were two significant correlations, although both weak. There was a weak correlation between the slope of the ranked bacterial community and measures of ECP. A less steep (more even) bacterial community correlated with more inflammation (rho 0.346; p=0.014). Additionally the percentage abundance of the most dominant species (be that *P. aeruginosa* or another species) had a negative correlation with ECP. This too suggests that a less even community is associated with less inflammation and *vice versa*. Finally species richness (RNA) and values for Neutrophil Elastase approached statistical significance (rho 0.227; p= 0.063). Were this to be a real correlation, then it too would favour the same direction of change between bacterial community and inflammation as those previously mentioned, namely a more diverse community being associated with more inflammation.

The low number of samples obtained per subject in each disease state, combined with many samples not collected pair wise before and after particular CFPEs, meant that a more detailed analysis of intra-subject variations could not be undertaken.

	DNA				RNA			
	Species Evenness			Species richness	Species Evenness			Species richness
	Slope	% of *Pa*	% dom. species	No. of species	Slope	% of *Pa*	% dom. species	No. of species
NE: rho	-0.104	0.157	0.096	-0.115	0.147	-0.086	-0.154	0.227
***p* value**	0.403	0.216	0.438	0.390	0.243	0.510	0.210	0.063
N.	67	64	68	68	65	61	68	68
IL-8: rho	0.064	0.067	-0.022	0.002	0.112	0.007	0.099	0.086
***p* value**	0.631	0.623	0.866	0.985	0.402	0.960	0.451	0.516
N.	59	56	60	60	58	53	60	60
ECP: rho	0.071	-0.059	-0.133	0.043	**0.346***	-0.172	**-0.340***	0.196
***p* value**	0.618	0.685	0.342	0.760	**0.014**	0.238	**0.013**	0.159
N.	52	50	53	53	50	49	53	53

Table 7-2 Correlation between measures of species evenness and richness and inflammatory mediators, using Spearman's rank correlation (rho). Correlation considered statistically significant if *p* 0.05 (*); N = number of samples

7.1.2 Inflammatory Mediators *vs.* Clinical Data

Visual analogue scales used in a longitudinal fashion in CF is novel. Sputum inflammatory mediators, although well established as a research tool, do not have a well defined role in clinical management or as a surrogate end point for clinical trials. It has been shown that inflammatory mediators are responsive to intravenous antibiotic therapy (Becker *et al.* 2009; Rottner, Freyssinet, & Martinez 2009; Teichgraber *et al.* 2008). However, a prospective evaluation of their relationship to symptoms has not previously been undertaken. As subjects provided frequent assessments of the clinical health using the CMF, which included the four VAS domains as well as spirometric readings, an assessment of the correlation between sputum inflammatory mediators and measures of clinical well being, through VAS scores and spirometry, was undertaken.

The results are presented in Table 7-3 below. It should be noted that samples taken on days for which there was no CMF (e.g. CMF/sample days Monday/Wednesday/Friday, but clinic visit on Tuesday or Thursday) were excluded from this analysis. When the analysis was repeated to include these excluded samples, with extrapolation of the nearest CMF results received (within +/- 2 days), all significant correlations disappeared (results not shown).

Between VAS values and sputum inflammatory mediators, there was no significant correlation. Between FEV1 and both IL-8 and Neutrophil Elastase there was a statistically significant negative correlation (rho -0.358, p=0.028 for FEV1 with NE; rho=-0.418, p=0.014 for FEV1 with IL-8). There was no correlation between ECP and any of the clinical measures.

As with other work based on the samples for sputum inflammatory mediators, there were insufficient samples per subject, and insufficient samples taken at appropriately paired time points before and after particular antibiotic therapy, to allow a more details analysis.

	Breathlessness VAS	Cough VAS	Sputum VAS	General Wellbeing VAS	Sum of VASs	FEV1
NE: rho	-0.215	-0.114	-0.075	-0.168	-0.171	-0.358*
***p* value**	0.196	0.495	0.654	0.314	0.304	0.028
N.	38	38	38	38	38	38
IL-8: rho	-0.249	-0.059	-0.067	-0.198	-0.165	-0.418*
***p* value**	0.156	0.740	0.705	0.261	0.352	0.014
N.	34	34	34	34	34	34
ECP: rho	0.156	0.179	0.164	0.229	0.182	-0.126
***p* value**	0.402	0.334	0.379	0.215	0.326	0.501
N.	31	31	31	31	31	31

Table 7-3 Correlation between clinical measures and sputum inflammatory mediators, using Spearman's rank correlation (rho); considered statistically significant if $p \leq 0.05$; N = number of samples.

7.2 Discussion

Sputum inflammatory mediators have been studied in CF previously (Al *et al.* 2009; Amitani *et al.* 1991). Ordonez *et al.* demonstrated that treatment with intravenous antibiotics for physician defined CFPEs was associated with a fall in IL-8 and NE, with a rise in FEV1, and a fall in density of *P. aeruginosa* or *S. aureus* by culture (Ordonez *et al.* 2003). Results presented here do not provide statistical support for this, as, although there were lower levels during stable periods than at CFPE they were not statistically significant. However the results here are not directly comparable as there were methodological differences,

namely samples compared in this study were taken at various time points throughout the 12 month study period that were either identified as periods of stability or at the time of CFPE, prior to commencement of antibiotic therapy. Ordonez *et al.* and others have previously noted that inflammatory mediators are poorly reproducible (Ordonez, Kartashov, & Wohl 2004). In their study they found large variations in IL-8 and Neutrophil Elastase in induced sputum samples in young stable CF children, in three samples obtained on three consecutive weeks. Many of the samples in the work presented here were obtained at intervals greater than the weekly intervals reported by Ordonez *et al.*. This intrinsic variability may contribute to the findings presented here. In other studies, which have found a significant change in inflammatory mediators with treatment for CFPE, minimising the time interval between sampling and maximising the intervention in that time, may have contributed to the significant results found by other researchers. It is worth noting the large degree of variation over time in clinical symptoms reported by the subjects of this study (see Section 4.2 above). Some of this variability may represent, to a greater or lesser degree, pseudo-exacerbations. The occurrence of such pseudo-exacerbations has the potential to impact on the results of inflammatory mediators presented here, some of which may not have been from time periods which were not as clinically stable as would first have appeared without the symptom information provided from the CMFs. Two other points of difference to note between this work and the work by Ordonez *et al.* are: 1) their sample size was 55 subjects and 55 CFPEs compared to exacerbations in only 12 subjects here; 2) all of the subjects in the Ordonez study received IV antibiotics, which is not true for the CFPEs presented here (Ordonez *et al.* 2003).

In addition to the comparison between inflammatory mediators at times of CFPE and clinical stability, comparison was made with results from the Clinical Monitoring Forms. These forms were completed by subjects at the time of producing samples for microbiological analysis, which often coincided with the collection of samples for inflammatory mediator analysis. This analysis showed a weak but statistically significant correlation between IL-8 and FEV1 (rho -0.418, p= 0.018) and between NE and FEV1 (rho -0.358, p= 0.028). There no significant correlations between ECP and any clinical measures, nor between any of the Visual Analogue Scale scores and inflammatory mediators. The correlation of FEV1 with NE is similar to that reported by Mayer-Hamblett *et al.* (Mayer-Hamblett *et al.* 2007) in a much larger cohort of 269 subjects, who reported a rho value of -0.35. They also reported a correlation between IL-8 and FEV1 (rho -0.28). Due to the larger cohort in their study, they were able to use the more robust confidence interval technique of boot strapping. It is also worth noting that their study reported correlations between FEV1% predicted rather than absolute FEV1.

It is shown here that there were no strong correlations between inflammatory mediators and bacterial community measures. In Table 7-2 above it can be seen that there were weak correlations between ECP and bacterial community slope. The direction of these changes suggest that, as the bacterial community became more even, there was more ECP. This finding is noted consistently, whether assessing slope, or proportion of the dominant species. There was no correlation with the proportion of *P. aeruginosa*. The nature of the connection between ECP and community is not clear. Koller *et al.* found a clear relationship between IL-8 and ECP levels in CF (Koller *et al.* 1997). However

IL-8 did not show the same relationship with RNA bacterial community measures as ECP here. It is possible that there are other explanations for the association, such as additional roles for ECP and eosinophils in CF, as yet to be elucidated. However, it is also possible that the association may relate to a genuine relationship between the bacterial community and ECP. Further studies would be needed to answer these questions.

There only limited association between measures of bacterial community structure and inflammatory markers is at odds with the pre-study hypothesis. Perhaps, however, it should come as little surprise, as there is growing evidence for the intrinsic pro-inflammatory nature of the CF airway in addition to inflammation that is driven by bacterial presence (Rottner, Freyssinet, & Martinez 2009). In addition, there are other drivers of airway inflammation other than bacteria. It has recently been noted that there is a very large diversity of viruses in the CF airway (Willner *et al.* 2009). A similar finding of previously undetected microbial diversity has been made when detecting of fungi from the CF airway using culture independent molecular techniques (Nagano *et al.* 2009). It should however be made clear that these findings are from single time point studies, and the clinical significance of such findings is yet to be ascertained. However, if the *bacterial* community is of importance in lung inflammation and damage, then either directly or indirectly, the wider microbial community is also likely to play a role.

Chapter 8 - Discussion

This study has attempted to extensively analyse some of the complexity inherent within the pathology of Cystic Fibrosis pulmonary disease. Previous attempts to understand the mechanisms of pulmonary exacerbations inCF lung disease and associated symptoms have met with partial success. The clinical relevance of *P. aeruginosa* detection by culture has been well described (Frederiksen, Koch, & Hoiby 1997; Littlewood *et al.* 1985), but targeted therapy has not been effective at altering established infection, nor explaining the heterogeneity of clinical outcomes (Liou *et al.* 2001). Bacterial diversity beyond those species easily detected by culture has been documented (Rogers *et al.* 2004; Rogers *et al.* 2008), but the relevance of such diversity to CFPEs, and the accepted dominance of *P. aeruginosa* has not been challenged. This study has attempted to move the scientific conversation forward from descriptive studies of diversity to a more in depth assessment and linkage of these changes to the underlying pathology.

The fourteen subjects in this study were a heterogeneous group, with wide variation in baseline lung function, genotype and diabetic state. Although patients who chose to participant in research studies may not be representative of socio-economic status across all CF patients (Goss *et al.* 2006), it was felt that in terms of a UK population the subjects were broadly representative of the type of patients seen by clinicians in adult CF clinics (Cystic Fibrosis Trust 2009b).

8.1 Clinical Monitoring

Patients' perception of symptoms is an important part of their lives with Cystic Fibrosis. Symptom reporting is integral in determining a clinicians decision to diagnose and instigate treatment for a CFPE. CFPEs have, in turn, been shown to independently predict long term (5 year) survival (Liou *et al.* 2001), and to correlate with quality of life (Britto *et al.* 2002). As above, there is still no accepted definition of what constitutes a CFPE, however (Marshall 2004). Most definitions are based on new symptoms or changes in pre-existing symptoms (Rosenfeld *et al.* 2001). See Figure 1-1. Few definitions take account of day to day fluctuations in symptoms that occur without recourse to intervention from a clinician. Such variability has not previously been quantified. The VASs used in this study fulfil most but not all of the criteria for a valid PRO, as laid out by Goss and Quittner (Goss & Quittner 2007). The area in which they fail the test is in the lack of a validated minimum clinically important difference (MCID). A.L. Ries (2005) attempted to estimate this for VASs, giving a MCID range of 10-20 based on studies of Pulmonary Rehabilitation in COPD. However, it can be seen in this review, that the MCID is dependant on the baseline starting value, with higher baseline values having a higher MCID and *vice versa*. Thus it is possible that each subject may need to set their own internal MCID, a strategy that would be impractical for most clinical studies.

The Visual Analogue Scales showed marked variability on a day to day basis in all individuals. However, when compared to each other there was good correlation between symptoms. For example, in Figure 4-3 above it can be seen that Cough and Sputum VAS scores have a very strong correlation with each other with an r value of 0.858. Symptomatically and physiologically these

two domains are closely linked, and thus this result is not unexpected although has not been previously reported. As such, they may not be considered independent variables, although hey are distinct symptoms in proposed definitions of CFPE (Fuchs *et al.* 1994; Ramsey *et al.* 1999). As any subject with extensive bronchiectasis coughs more they, in turn, expectorate a greater volume of sputum and *vice versa*. The General Wellbeing VAS score correlated best with the Breathlessness VAS score (r = 0.733), which reflects the importance of exercise capacity and dyspnoea on quality of life. Increased cough or sputum, with less strong correlations with general wellbeing, affects QoL less than does breathlessness. The weaker correlations between the more specific respiratory symptoms of cough and sputum with GWB suggest that these symptoms have less impact on day to day quality of life. This partly agrees with other studies, which have found exercise capacity, along with sputum output, accounts for 54% of the variance in QoL as measured by the Chronic Respiratory Diseases Questionnaire (Bradley, McAlister, & Elborn 2001).

On inspection of the individual Response Profile Plots for each subject (see Appendix - 9.4 below), it can be seen that in these are marked day to day variability. Given the lack of visual anchors available (for example, there were unable to see how they had scored on the previous CMF, as it had already been collected by the courier), it is perhaps not surprising to see such variation. However, there are trends that emerge. A number of subjects have recorded changes in their symptoms that are of the same magnitude as other times when they received treatment for a CFPE (e.g. Subject 01, ~day 300). Subsequent to both diagnosed CFPEs and 'pseudo-CFPE' events there is a return to a

previously lower level, which has lead to these untreated deteriorations in symptoms to be termed as pseudo-exacerbations. Such a phenomenon is not new in respiratory illnesses. Wilkinson *et al.* (Wilkinson *et al.* 2004) described 1077 exacerbations in COPD of which only 658 were reported to a physician. Their study used simpler Likert scale-type diary monitoring than the VASs employed in this study, with all the advantages and disadvantages previously discussed. The Wilkinson study demonstrated that COPD exacerbations that were not reported to a physician took longer for symptoms and lung function to return to baseline, and were more likely to be hospitalised for treatment for their illness. The mechanisms behind such pseudo-exacerbations are unknown. It is possible, given the microbial lower airway colonisation that is a frequent and important feature of both diseases, that there may be similar processes at work in both. However, as yet the cause of many exacerbations remains unclear, and more work is required.

There is undoubtedly a significant psychological and social element in a patient's perception of their symptoms (Conway *et al.* 1996). For example, some of the subjects in this study were in part time or full time employment or education (Subjects 01, 05, 06, 07, 08, 10, 12). Subject 01 was a teacher, and may have been somewhat reluctant to seek medical assistance during school term time, and may try to time treatment for CFPEs with school holidays or half term (Hodson & Geddes 2007).

Lung function is an important determinant of outcomes in CF, although it is recognised that is not a key component in the diagnosis of CFPEs (Rosenfeld *et al.* 2001). By inspection of Table 4-2 above, it can be seen that there is a range of strengths of correlation between symptoms and FEV1. This ranged from a

strong correlation (r = -0.565, p<0.001) for Subject 13, to no correlation at for Subject 12 (r = -0.086, p=0.376). Such correlations have not previously been described in such a way. For those without a correlation, it may be worth exploring some explanations. Subject 09 will not be discussed here as there were insufficient paired data points to allow a valid comparison to be made (N=3; see Table 4-2). For Subject 08, it is necessary to review the individual RPPs in the Appendix (See Section 9.4.8 below). Through the time of the study, the individual RPPs for Cough and Sputum both show a gradual improvement, but General Wellbeing and Breathlessness showed a gradual worsening. During this time FEV1 remained remarkably stable. Thus, it appears that there may have been other factors at play in influencing the changes seen in the VAS and the Sum of VAS scores. It is possible that there was a lack of recall in how this subject rated their symptoms. Perhaps, over time, this subject's perception of their previous breathlessness was diminished. Yet the opposite occurred with cough and sputum. It is of course possible that the changes recorded were in fact measuring a real change in symptoms, not detected by FEV1, and not explained by symptom domains captured by these VASs. There is evidence that changes in spirometric values are an insensitive marker for underlying lung damage when compared to Computerised Tomography of the lungs (de Jong *et al.* 2004).

The other study subject, in whom no significant correlation between Sum of VAS scores and FEV1 was seen, was Subject 12. Although Subject 12 was in higher education, their first language was not English. In addition to this, there were a number of prolonged periods during the study where they were not resident in the UK, and were not recording symptom scores or providing sputum

samples. These factors may at least in part explain why this subject did not show the expected correlations.

The use of FEV6 as a surrogate measure of the vital capacity, as used in this study, is not universally accepted (American Thoracic Society 1991). However its use in this way offers a number of important potential benefits. Firstly, it is reported to be more reproducible than Forced Vital Capacity (Swanney *et al.* 2000). In addition, it offers a reduced duration of effort for subjects with significant airflow obstruction. Finally it offers a more objective and explicit marker for the end of the test. It has been shown to be equally valid to FVC in measurement of airflow limitation in COPD (Akpinar-Elci, Fedan, & Enright 2006), although its use in CF does not appear to have been previously reported. There were correlations seen with symptoms as measured by Visual Analogue Scales (see Table 4-2 above). In 11 Subjects there was a significant correlation between spirometric values and VAS scores; in 4 of these 11 the correlation between FEV6 and symptoms was stronger than the correlation between FEV1 and symptoms. This suggests that FEV 6 is a valid marker of symptoms, but may not be as good as FEV1.

8.1.1 Limitations of Clinical Monitoring

Most symptoms are most accurately described as continuous rather than discrete variables. Despite this many longitudinal studies of lung health use categorical variables to capture data about pulmonary exacerbations (Seemungal *et al.* 2000; Wilkinson *et al.* 2004). The most obvious advantages of this categorical approach are that analyses are somewhat simpler and some of the subjectivity is removed. By removing the part of the subjectivity, it allows

a more valid comparison between subjects. The use of Visual Analogue Scales is well described. Gift *et al.* (Gift 1989a) validated their use for the assessment of dyspnoea in asthma and COPD, whilst Smith *et al.* (Smith et al. 2006b) demonstrated good correlation of a cough VAS to objective sound recording measures of cough. In addition to the validated use of breathlessness and cough VASs in this study, sputum and 'General wellbeing' VASs were used, which to the best of knowledge, have not been previously validated. However the internal consistency of the measures was demonstrated (Section 2.1.4.2 above), and strong correlations were seen between the different symptom domains. See Figure 4-3 above. The fact remains, though, that VAS data were highly variable both within and between subjects. This can be best seen in Figure 4-1 above. Overall the median (IQR) for breathlessness VAS was 20(19). These results can be compared to others (best summarised in Ries, A.L. (2005)). In interventional studies in COPD, Reardon *et al.* found a mean (±SD) baseline VAS of 74.4 (±18.9), whilst Alvisi *et al.* found values of 25.0 (± 18) (Alvisi *et al.* 2003; Reardon *et al.* 1994). With regard to Cough VAS, the results presented here showed a median Cough VAS of 22 (IQR 22). Smith *et al.* showed similar results, with a median Cough VAS of 18 (IQR 41) after treatment for a pulmonary exacerbation. However no study has shown the longitudinal variation over time in CF as has been presented here. The form of the data captured allows insight in to the day to day fluctuations in symptoms which to a very large extent go unreported to the clinical team.

To quantify and compare the changes in symptoms between subjects is clearly going to be skewed by subjects who report a wider range and higher values of VAS. However, the alternative approach would be to in some way standardise

the range of responses seen in different subjects. One way to do this would have been to standardise raw VAS scores by converting the minimum score for a particular subject's VAS to zero%, and converting the maximum score to 100%. This too comes with considerable statistical problems. For example, it would mean that, in subject 13, a single point change in Breathlessness VAS would equate to a ((1/3)x100=) 33% rise in breathlessness, compared to a single point change in subject 10 equating to a ((1/28)x100=) 3.6% increase in relative breathlessness. There was no precedent identified in the literature for normalising or standardising VAS scores, and thus the decision was made to work with the raw scores. In addition changes in VAS scores only weakly correlated with the occurrence of CFPE. This may be a weakness in the VAS method of monitoring, in so much as it is inherently going to have large intra- and inter-subject variability, but may also truly be a reflection of the wellbeing of the subjects over time.

There are no validated definitions of what constitutes a CFPE. In common with a number of other observational studies (Ordonez *et al.* 2003; Sibley *et al.* 2008c), a clinician-based definition of a CFPE was used here. Many other studies have only defined CFPEs for which intravenous antibiotics have been used. It was the practise in SGH to use oral antibiotic therapy for many CFPEs, thus reducing the treatment burden for patients and conserving the greater health resource that is utilised when treatment is with intravenous therapy. Although this allowed the analysis of many more CFPEs than would have been possible if analysis had been limited to IV courses, it means that many of the CFPEs studied may have been less severe than those in other cohorts (Ordonez *et al.* 2003; Smith *et al.* 2003).

Much consideration was given to which symptoms and with what wording should be included for monitoring in the Clinical Monitoring Form. With hindsight, there were two additional areas that could have been incorporated to provide a more robust assessment of pulmonary health in CF. There were occasions when haemoptysis was the precipitant for treatment for a CFPE, and the CMF provided no mechanism for capturing these data. In addition there are changes to the qualities of sputum, beyond its volume, that contribute to clinical decision making. This is apparent in the subtle difference in wording seen between the definitions of CFPE used by Fuchs *et al.* (1994), and by Ramsey *et al.* (1999) with regard to the role of sputum in CFPEs (see Figure 1-1 above). The earlier study by Fuchs *et al.* includes the less specific 'Change in sputum', whereas the latter study by Ramsey *et al.* includes the more specific 'Increased sputum volume (increase of 50%)'.

There are additional signs and symptoms which may be important in indicating a pulmonary exacerbation that were not included in the clinical monitoring. These included sputum viscosity (Serisier *et al.* 2009), sputum colour (Anthonisen *et al.* 1987), and taste (Barker *et al.* 2006). However, given the repetitive and long term nature of the study, the inclusion of such additional information for subjects to complete would have had a significant negative impact on the time required. Hence the likelihood of study protocol adherence would have been diminished (Kehoe *et al.* 2009).

There are results from some subjects that require additional discussion to allow a fuller understanding of their results.

Subject 04

This subject was enrolled in to the study with the understanding that they were regular and frequent sputum producers (see Inclusion Criteria Section 2.1.3). However it can be seen from the sampling frequency from this subject that the minimum sampling frequency was not achieved, with only 43 samples in 52 weeks. After enrolment and the start of the study it became apparent that they did not expectorate sputum on most days, but at a much reduced frequency, averaging les than one sample per week, and fewer when there were no exacerbations. Because the study was designed so that clinical monitoring only occurred when a sputum sample was produced, this resulted in a very hit and miss record of their symptoms and microbiology, in comparison to the other subjects. This reduced sampling frequency, with a potential bias towards sputum samples at the time of exacerbation had a negative impact on the quality of the results from this subject.

Subject 09

Subject 09 also deviated from the study monitoring protocol, but for very different reasons to Subject 04. These are now discussed in more detail.

The Clinical Monitoring Form was tested for subject acceptability by patients receiving in patient care, with positive feedback about the ease of use. However, it became apparent during the study that one Subject (09) was having difficulty in completing the CMF correctly. The VAS results from Subject 09 were all reporting no or minimal symptoms in any VAS domain, and yet it was known, clinically, that Subject 09 was severely disabled by their lung disease. Subsequently a meeting was held with Subject 09, who confirmed that the

VASs were being completed incorrectly. After further training on the process all future VASs were adequately completed. The decision was taken to exclude all VAS data from Subject 09 prior to the re-education (about 6 months of data). As well as the direct loss of data, this was unfortunate for two other reasons. It was at about the same time that this Subject stopped performing CMF spirometry, due excessive dyspnoea (FEV1 often recoded as <0.5L). In addition, due to frequent CFPEs, this Subject had already been chosen as one whose samples would be analysed more extensively. It was thus not possible to link microbiological changes to symptom scores so well (see Figure 4-20 above).

Thus two of 14 subjects provided sub-optimal data, but 12 of 14 provided acceptable data. It is known and accepted that incomplete adherence to clinical trial protocols occurs. For example there was a 10% drop out rate for subjects in a 6 month RCT of inhaled tobramycin in CF (Ramsey *et al.* 1999).

8.2 Sputum Culture

Previous work has suggested a role for bacteria from the *Streptococcus milleri* group (SMG) (Sibley *et al.* 2008b). Bacterial sputum culture detected bacteria from Group F streptococci, which includes the SMG. However, in the one subject from who it was identified, no temporal correlation with the occurrence or treatment of CFPE cold be discerned.

The number of bacterial species detected and identified by culture remained stable over time at just less than two per sample (see Figure 5-4 above). This is slightly fewer than the 2.8 pathological species detected per sample in a large

cohort of American CF patients (Burns *et al.* 1998), but the data are not directly comparable as the American results included fungal species in the count.

8.2.1 Limitations of Bacterial Culture

The limitations of selective bacterial culture in its ability to discern bacterial diversity in CF sputum has been well described (Rogers *et al.* 2004; Rogers *et al.* 2008). The methods employed in this study were a little different to those employed in other studies that employed bacterial culture. Overall this study looked to undertake bacterial culture in a manner identical to that used in every day clinical practice (Cystic Fibrosis Trust 2001), which uses semi-quantitative estimates of bacteria detected. Other studies have used quantitative methods, such as colony forming units (Regelmann *et al.* 1985; Regelmann *et al.* 1990). In addition, although not a weakness of culture *per se*, fungal cultures were performed. This was not mirrored in the methodology in the culture independent work, due to the different molecular techniques required.

8.3 Inflammatory Mediators

Significant correlations were seen between the pro-inflammatory cytokine IL-8 and changes in FEV1, in keeping with the findings of others (Mayer-Hamblett *et al.* 2007). Given the underlying pro-inflammatory state of the infected CF lungs this is not unexpected. However, values of NE, IL-8 and ECP were not statistically different at a clinically stable baseline compared to the start of a CFPE. Possible explanations for this surprising negative finding are discussed above.

The lack of association between inflammatory markers and measures of symptoms is interesting. It might have been expected that more inflammation would have been associated with worse (higher values) symptom scores. More inflammation is certainly associated with significantly worse lung function, which in turn is strongly linked with worsening dyspnoea. However, the data presented here have failed to complete the triangle. This may be because inflammation does not directly drive dyspnoea, and the only indirect link between the two is the effect of inflammation on worsening lung function through tissue breakdown (Hilliard *et al.* 2007). In fact this is more biologically plausible than inflammation itself having a direct causal link to dyspnoea. Despite this, if the sampling size was large enough one still might have expected to find an association between inflammation and symptoms, even if only because increased inflammation is associated with CFPEs, without implying a direct causal link. However, the nature of the VAS as a tool to measure symptoms is intrinsically subjective and, between subjects, scores ranged without a clear link to their underlying disease severity. Taken together, this would suggest that the VAS is not a tool that is well suited to this kind of between-subject analysis.

In the comparison of inflammatory mediators with measures of bacterial community structure, there were only two results of significance. Both related to ECP, a little studied protein in CF. Previous findings of raised levels of ECP correlating with CF disease severity were in themselves surprising, as eosinophils are not a feature of the CF airway (Koller *et al.* 1996). No relationship to bacteriology has previously been noted. The significance of the current findings is unclear. It may be that the host immune responses to the

changes in the bacterial community structure are led by an eosinophilic driven process. Although the association is not strong, the direction of change is in keeping with results from other parts of this thesis, which have noted that there a more even community is seen during treatment for CFPE than before treatment.

8.3.1 Limitations of Inflammatory mediators

Inflammatory mediators were collected from the 14 subjects at various time points throughout the study period. Subjects gave a median of 5 samples (range 2-10). Samples were given at times of clinic attendance, which limited the ability to obtain samples at the optimum times around CFPEs. It was not possible to obtain paired samples before and after a CFPE. This limited the ability to correlate changes in inflammatory mediators that would be expected as a result of antibiotic treatment (Ordonez *et al.* 2003). Changes seen in inflammatory mediators from times of CFPE to times of clinical stability may have had many other events between the intervention and the measurement that would make any change less reliable. As can be seen from the results of symptoms using the clinical monitoring forms, there may be pseudo-CFPE, or other inflammatory airway events that were not diagnosed as CFPE.

It was unfortunate that the degree of inflammation in some subjects was unexpectedly high. This meant that some results, that could not be repeated, fell above the upper limit of the standard curve and thus could not be accepted as reliable results. This negatively impacted on the results of inflammatory mediator measurements.

8.4 Culture independent bacterial analysis

8.4.1 Periodic Stable Sampling

Periodic stable sampling was undertaken in an attempt to understand what happens to the bacteria in the CF airway during the study period, without the periods of what were hypothesised to be the greatest fluctuations, CFPEs. Such a large scale longitudinal analysis of bacteria in the CF airway has not previously been attempted, although longitudinal analysis of bacteria by culture independent techniques in the human gastrointestinal tract (De La Cochetiere *et al.* 2008; Dethlefsen *et al.* 2008; Mackie, Sghir, & Gaskins 1999), and other non-clinical settings have been performed (Bell *et al.* 2008; Hongoh *et al.* 2005; Robinson *et al.* 2009). The findings presented here show a high degree of bacterial community stability over time, with 10 of 14 having the same basic community of bacteria (*i.e.* the same dominant species in the top few positions of the species rank abundance plot) at the end as at the beginning. This is despite, or possibly because of, the panoply of antibacterial treatments received by the subjects of the course of the study. It would be well accepted by most clinicians that *P. aeruginosa* becomes dominant in the airway of most CF patients and would remain permanently as such (Cystic Fibrosis Trust 2009a), eradication attempts not withstanding (Taccetti *et al.* 2005). Thus, by culture based techniques *P. aeruginosa* would achieve stability over time. However, the result presented here show more than stability of one species, but of a community of bacteria. In a number of subjects species or bands other than *P. aeruginosa* achieve the same level of stability over time (*e.g.* subject 01). In addition the species further down the rank abundance curve are also relatively stable. Such stability over time has been seen in other environmental

communities, despite marked temporal perturbations (Lacap, Barraquio, & Pointing 2007). There a number of possible explanations for this. This might suggest that the established community of bacteria has not been challenged by new species. This seems unlikely, given the widespread abundance (Curtis, Sloan, & Scannell 2002) of microbes in the mouth (Aas *et al.* 2005; Socransky *et al.* 1963), and in the air (Fierer *et al.* 2008; Wainwright *et al.* 2009). Others have assessed factors responsible for colonisation of new and established biofilms, albeit in an *in vitro* stream model, and found that both physical and biological factors influence the likelihood of colonisation (Augspurger *et al.* 2010). It thus seems likely that the bacterial community has some intrinsic stability, in which it has some characteristics that make it resistant to intrusion by other bacteria. In terms of biological reasons for this, it may be due to limitations in supply of key nutrients, or substrate, upon which a given bacteria is dependent in order to make a living. Availability of nitrate has been shown to affect the gene expression of *P. aeruginosa* (Hoffman *et al.* 2010), whilst the ability to utilise iron may be important for bacterial fitness (Harrison & Buckling 2009). There may also be physical barriers, in the amount of secretions in the lower airway that prevent new bacterial species entering the established bacterial biofilm communities that exist (Donlan & Costerton 2002). The clinical significance of stability, and lack of it in some subjects, is not clear. it is possible that the stability is, in part, driven by the antimicrobial therapy, with inhaled aminoglycoside therapy (Ramsey *et al.* 1999) resulting in increased propensity of bacteria such as *P. aeruginosa* to form biofilms (Hoffman *et al.* 2005)

From the ribosomal gene clone sequence analysis it is possible to accurately establish that a considerable proportion (44%) of the bacteria in the CF lower airway are obligate anaerobes (see 5.1.1.1 above and Table 9-1 below). This is in keeping with a number of previous studies that utilised both culture dependent and culture independent techniques. Rogers *et al* (Bittar *et al.* 2008; Rogers *et al.* 2004; Rogers *et al.* 2005; Rogers *et al.* 2008; Tunney *et al.* 2008). Tunney *et al.* described large numbers of anaerobes by culture, with anaerobic species in 42 of 66 samples (64%). Using 16S bacterial clone sequence analysis Bittar *et al.* identified 736 clones, of which 119 (16%) were considered obligate anaerobes.

In addition to bacteria that are only capable of anaerobic respiration, there are some species which have been shown to be facultative anaerobes in the CF airway, e.g. *P. aeruginosa (Worlitzsch et al. 2002)*. This has potentially important implications in a number of ways. Firstly, antibiotic strategies that target *P. aeruginosa* alone, without targeting other anaerobic bacteria will not provide optimum anti-bacterial spectrum cover. In addition, culture based antibiotic sensitivity testing results, are based on aerobic culture of *P. aeruginosa* cells not growing as a biofilm. It has been previously shown that *P. aeruginosa* in a planktonic form is intrinsically less resistant to antibiotics than the biofilm form that is more akin to the *in vivo* mode of growth (Hill *et al.* 2005). Additionally, it opens up the possibility that there may be other ways to add physiological stress to the growth of anaerobic bacteria beyond the use of antibiotics and the associated side effects (Cystic Fibrosis Trust 2009a). One possible therapy that may be worth future study is supplemental oxygen. Increased oxygen concentrations in the CF airway would reduce the amount of

anaerobic secretions in which anaerobic bacteria flourish (Worlitzsch *et al.* 2002). Supplemental oxygen is already used to reduce complications of advanced CF lung disease associated with chronic hypoxaemia (Cystic Fibrosis Trust 2001). In addition to these potential benefits there is evidence that alveolar hypoxia may contribute to the pro-inflammatory milieu of the CF airway (Leeper-Woodford & Detmer 1999). Thus the use supplemental oxygen may theoretically have a role to play in reducing the vicious cycle of inflammation and infection, as well as perturbing the anaerobic bacterial community.

The presence of such large number of bacterial species that are associated with the human oropharynx is of interest. They have been shown to be not just 'passing through', but members of the core community of bacteria. This is an important point, as Magurran and Henderson made clear in 2003 (Magurran & Henderson 2003). In their paper, which studied fish species and number in the Bristol Channel over a twenty year period, they showed that it was possible to differentiate between species that are transient in the sampling environment, and those that are resident species. In the context of the CF lung and the mucociliary escalator, these resident species are analogous to the core species of the lung, and the satellite species are analogous to the transient fish species that were found in the Bristol Channel after storms out in the Atlantic Ocean. This raises another question. What is the source of these two populations of lung bacteria? Both core and satellite communities have 'oral' bacteria in them. It is known that the mucociliary escalator is a crucial part of lung host defence (Regnis *et al.* 1994), and that clearance is impaired in CF (Robinson & Bye 2002). In addition it has been shown that micro-aspiration of gastro-oesophageal contents occurs in to the CF airway in significant amounts (Ledson

et al. 1998; Ledson, Tran, & Walshaw 1998b). Finally the mechanisms that drive whether a species moves from transient to resident, satellite to core species has been shown to be at least partly stochastic (van der Gast, Ager, & Lilley 2008). Thus, this situation combines the predisposition of the CF airways to persistent infection, the ongoing mechanism by which such oral and gastro-intestinal bacteria could reach the lower airways and evidence for why some become core species and others do not. The work presented here demonstrates that such bacteria, which have previously not been considered as part of the usual CF respiratory bacteria and only demonstrated as present by molecular techniques in single time point studies, are present over long periods.

A number of different measures were employed to elucidate differences in bacterial community structure in repeated samples from periods of stability during the study (Section 5.2). A number of significant findings were made. In Section 5.2, it was shown that subjects with more severe CF pulmonary disease, as determined by FEV1 percentage predicted, had significantly more uneven bacterial communities and fewer species detected. This could be explained in a number of ways. Firstly, FEV1% is an independent risk factor for CFPE (Block *et al.* 2006). Thus, those with more severe CF pulmonary disease are likely to have had more frequent courses of antibiotics, as well as more lifetime antibiotics. In turn, these antibiotics may have applied selective pressures to the bacterial community resulting in a reduction in the number of persistent species, increased dominance of those species that did persist and a commensurate increase in the unevenness of the bacterial community. An additional explanation for this finding, and not necessarily one in which is mutually exclusive to the first, is that a less even community is one that results

in greater potential for lung damage. Support for such an idea comes from *in vitro* work on *P. aeruginosa* and decreased virulence in the presence of a more diverse community (Harrison *et al.* 2006). Thus the presence of such an uneven community and reduction in bacterial diversity may be a contributory factor to the pathological process.

In addition to differences in the bacterial community between subjects with high and low lung function when assessed by bacterial community evenness, differences were seen between the groups when assessing similarity between samples. A significant difference was seen when assessing using the Bray-Curtis Index. There was a greater difference between consecutive samples with lower lung function, a finding that would be consistent with the theory that different parts of the more damaged and ecologically heterogeneous lungs harbours distinct bacterial communities. This would offer an explanation as to why Subjects 5 and 9 had Periodic Stable T-RFLP plots that were so much more variable than other subjects. However, this theory would be out of keeping with previous work (Gutierrez *et al.* 2001), where multiple lobes of CF lungs under went bronchoscopic lavage. However, this work was culture based and thus not comparable to the work presented here. Furthermore the comparability of such findings is further hampered as the work was in children (age range 1.5 – 57 months).

In addition to differences seen between subjects grouped by lung function, there were significant differences between subjects grouped by gender. This is an area of interest to clinicians, as there is an as yet unexplained difference in survival between females and males with CF (Corey *et al.* 1997; Liou *et al.* 2001; Rosenfeld *et al.* 1997). In this work there was a significantly more

uneven bacterial community in female subjects than in males. This is despite the males having on average a lower FEV1% (49% vs. 58%), which has been shown to favour a steeper slope. There other baseline characteristics and factors not accounted for in this association, such as course of antibiotics, baseline therapy, and adherence to therapy to name but a few, but this is a new finding and warrants further attention. In addition to the differences in bacterial community evenness, differences in the data emerging from the similarity indices used was noted. There was no difference in similarity when measured by the Sorensen Index, but there was a difference when the Bray-Curtis Index was used (see Figure 5-13). This disparity between the two indices is perhaps unsurprising as the Sorensen Index is a less sensitive measure of bacterial community fluctuations as it uses binary species abundance data (*i.e.* species present or absent), whereas the Bray-Curtis Index uses quantitative proportion of bacteria present in each sample. The use of the Sorensen Index helped to detect the presence (or lack) of new species in the community. Given the stability of species present in the communities over time, yet fluctuations in proportions of bacteria present, it is perhaps no surprise that the Bray-Curtis Index appears more sensitive than the Sorensen Index to detecting changes. It remains possible that the difference in slope between genders may contribute to the cause of the gender mortality gap through being associated with more inflammation and lung damage. It is equally possible that it is due to a third, as yet unaccounted for factor that is responsible for the mortality gap and the difference in bacterial community evenness. It has previously been suggested that hormonal differences may play a role (Gurwitz *et al.* 1979). More recent studies have refuted this however, as the mortality gap was at its most pronounced before puberty (Rosenfeld *et al.* 1997). The Rosenfeld database

study from 1997 (Rosenfeld *et al.* 1997) examined over 20,000 American CF patients, and no associations to explain the gender gap were found. Only one study has suggested a microbiological explanation for the gap (Demko, Byard, & Davis 1995). Demko *et al.* found that females had earlier acquisition of mucoid *P. aeruginosa* , but both males and females had the same rate of decline in lung function after acquisition. In addition the gender mortality gap persisted even among subjects with only the non-mucoid form of *P. aeruginosa*. The authors concluded that factors other than *P. aeruginosa* may play a role. The work presented here offers new insights in to possible linkage between bacterial community structure and the gender mortality gap in CF. It is also of interest that the mortality gender gap may not be limited to CF, as recent work has suggested that females with COPD may do worse than males (Ekström, Franklin, & Ström 2010).

Another finding with potential significance is the observation that there is a association between the presence of band 268 ('Unknown species 17') and subjects being frequent exacerbators (Figure 6-13 above. This finding is of great potential significance, particularly as this band does not appear to be *P. aeruginosa*. If confirmed by larger studies, it would offer the hope that targeted therapy at this species might reduce rates of CFPEs with all the associated negative health, QoL and cost implications (Britto *et al.* 2002; Liou *et al.* 2001; Weiner *et al.* 2008). As such it is unfortunate that clone sequence analysis, undertaken on one sample from each subject at the start of the study, did not reveal any species that may have been responsible for band 268. It is thus of great importance to identify the origins of band 268.

8.4.2 Culture Independent Microbiological Analysis of CFPEs

The cause of CFPEs is as yet unknown. In COPD, exacerbations have been linked to the acquisition of new bacterial strains, albeit bacteria grown from sputum by culture based techniques (Sethi *et al.* 2002). It has previously been suggested that CFPEs may be due to the acquisition of new strains of *P. aeruginosa*, but there is now convincing evidence that this is not the case (Aaron *et al.* 2004). It is likely that, if there is a proceeding trigger that causes the change in respiratory symptoms in CF, it is multi-factorial (Goss & Burns 2007).

When reviewing the effects of antimicrobial therapy on airway microbiology in CF, the evidence for clear benefit is not as convincing as might be assumed (Wolter, Bowler, & McCormack 1999). *P. aeruginosa* is consistently found by culture before and after both IV antibiotic therapy targeted at *P. aeruginosa* (Gold et al. 1985; Gold et al. 1987; Regelmann et al. 1990) at CFPE, and during long term oral (Saiman *et al.* 2003; Wolter *et al.* 2002) and inhaled antibiotics (Mccoy *et al.* 2008; Ramsey *et al.* 1999; Retsch-Bogart *et al.* 2009) in chronically *P. aeruginosa* infected subjects. There is thus little evidence to suggest that antimicrobial therapy is 'treating' these pulmonary infections in the same way that an acute infection in a sterile environment is treated. Thus antimicrobial therapy for pulmonary exacerbations in CF may be having alternative or additional modes of action beyond an effect of *P. aeruginosa*.

The results presented here cast more light on the "murky" world of bacteria in the CF airways before, during and after CFPEs. Others has previously identified significant quantities of anaerobic bacteria in cohorts of CF subjects (Bittar *et al.* 2008; Tunney *et al.* 2008; Worlitzsch *et al.* 2009). Figure 6-2 and

Figure 6-4 above show changes in bacterial community richness and evenness around the time of CFPE. The mean number of species detected at day zero (prior to antibiotic treatment) is lower than at the time points measured in the preceding three weeks. This reduction in diversity is maintained for the duration of the treatment and beyond, although significantly outlying results were observed. Figure 6-7 shows that bacterial community evenness, as measured by the slope of the rank abundance for curve, also changes in a similar pattern. At day zero (prior to treatment for CFPE) the slope becomes steeper, a change that is reflected by the dip in FEV1 that occurs at around the same time. This change in slope would be expected, given the aforementioned reduction in diversity seen mentioned above. However, the introduction of treatment including antibiotics for the CFPE, do not then alter this reduction in richness and evenness. The changes also typically persist after the end of treatment. It would suggest that the reduction in diversity may play some role in the aetiology of the CFPE rather than being a response to treatment. However this reduction in diversity *prior* but not more than 6 days before treatment may be at odds with the change in symptoms that appears to occur a number of weeks prior to the start of treatment (see Table 4-18). After the end of treatment, lung function continues to improve, but no consistent changes are seen in bacterial community evenness and richness, although levels remain lower than in the three weeks leading up to treatment.

Other researchers have investigated the effects of antibiotics on bacterial communities, with conflicting results. Recently, and with most in common to the short term longitudinal effects studied here, Robinson *et al.* (Robinson *et al.* 2009) looked at the effects of antibiotics and nutrient changes on the bacterial

ecology of the Cabbage White butterfly larval midgut using culture independent strategies. Their study showed that caterpillars ingesting antibiotic impregnated feed resulted in a marked increase in the means species richness (from 14 to 43 species). This suggested that antibiotics were allowing the detection of more species by suppressing the previously most dominant species. The results from the CF airway presented here are at marked odds to this finding. However this may be, of course, because of the marked physiological and ecological differences that exist between the healthy Cabbage White butterfly larva, and the CF lung. Firstly, the caterpillar gut is an environment that has evolved, like all gastro-intestinal tracts, to harbour a normal community of bacteria that co-exist symbiotically with the host. The healthy gut does not mount an immune response to a normal bacteria community, in contrast to the CF lung which is in response, at least partly, to the presence of bacteria (Armstrong *et al.* 1997). A second important difference between the two situations is that antibiotics in CF were given in response to some change in the clinical situation, which may in turn have been driven by some change(s) in the bacterial community. Finally the CF lung is not an antibiotic naïve environment, but in fact quite the reverse, with continuous oral and topical (inhaled) antimicrobial therapy the norm in most cases, plus episodic IV antibiotics over many years. This will already have had major effects on the bacterial community structure for many years, and this study is assessing the situation many years after the first antimicrobial intervention.

Given the previous controversy around the effects of antibiotics on *P. aeruginosa (Wolter, Bowler, & McCormack 1999)* and other bacteria species (Worlitzsch *et al.* 2009) during treatment for CFPEs, work was done to assess

the effects of treatment during this study. In Figure 4-32 and Figure 4-33 above, it can be seen that treatment was clinically effective with returns to baseline levels in spirometry and symptom scores. The effects of treatment on key airway bacteria is shown in Figure 6-8 and Figure 6-9 above. For example, across the 12 CFPEs analysed, the proportion of *Prevotella* spp. prior to antibiotics was 25%, which had fallen to 6.35% by the end of the antibiotic course, whist the proportion of *P. aeruginosa* was 42.8% prior to antibiotics, compared to 68.1% after antibiotics. Here it was shown that therapy for CFPEs is preferentially reducing the proportions of anaerobes whilst increasing the proportions of *P. aeruginosa*. It is of interest there is a non-significant trend towards an increase in proportions of anaerobes in the 21 days preceding the start of CFPE treatment. In addition, the effects of treatment in reducing anaerobe proportions persist beyond the end of treatment. It is worth speculating whether there was a rise in the relative abundance in the anaerobic population even further before each CFPE, perhaps even leading as far back as the last systemic antibiotic course. In addition to the suggestion of a disproportionate effect of treatment on proportions of anaerobes, it should be noted that the proportion of *P. aeruginosa* rises during treatment, a finding that is counter to any that is seen in studies that have employed culture based techniques. Regelmann *et al.* (1990) showed a reduction in density of *P. aeruginosa,* as measured by colony forming units (cfu), attributable to antibiotic treatment alone. This fall was proportional to clinical improvement. Are these two pieces of work contradictory or is there a unifying explanation? Both techniques offer only a relative change in bacterial quantities, as there is no indirect way of quantifying the total number of bacteria within the lungs. Both techniques attempt to take account of bacterial cell viability, T-RFLP by using

PMA (Rogers *et al.* 2008) and culture by the fact that only live cells can form colonies. However, it may be possible to reconcile the two findings. It is possible that the total bacterial load per aliquot of sputum has been reduced by antibiotics (including the total *P. aeruginosa* content), but that a proportionately greater fall in the amount of anaerobes has occurred, as T-RFLP reports a percentage of a species in the total community.

This finding of a significant effect of treatment of proportions of obligate anaerobes is contrary to the only other longitudinal study of anaerobes during CFPE treatment. Worlitzch *et al.* (Worlitzsch *et al.* 2009) study anaerobes in sputa in 12 adult subjects with CF by using culture based techniques. They found no fall in quantitative colony counts of anaerobes between start and end of therapy for CFPE. The subjects in their study also received IV antibiotics in hospital or at home. Such differences in findings may well reflect the techniques used for detection and identification of anaerobes. For *P. aeruginosa,* culture based techniques have been shown to be poorly reproducible and of doubtful clinical significance (Foweraker *et al.* 2005; Smith *et al.* 2003). Given that many of the same short comings of selective culture techniques apply equally well whatever the species being cultured, this is perhaps no surprise.

It is worth mentioning that it is not possible from the current work to demonstrate what has driven the changes in bacterial communities that occur during treatment for a CFPE. Treatment consists of much more than just antibiotic treatment. Airway clearance techniques, nutrition, improved adherence to other prescribed therapies and supplemental oxygen therapy are all likely to play some part. Indeed oxygen therapy is worth further discussion.

It is well described that bacteria in the CF airway live in an oxygen depleted environment (Worlitzsch *et al.* 2002; Yoon *et al.* 2002). In the work presented here it has been shown that proportions of obligate anaerobes, including *Prevotella* sp fall during treatment, that often includes supplemental oxygen therapy in the acutely hypoxic patient (Cystic Fibrosis Trust 2001). It is therefore of great interest that there is *in vitro* work to show that oxygen may be directly toxic to *Prevotella* species (Takumi *et al.* 2008) as well as to strict anaerobes generally. As a result of the findings presented here, and the published work of others, new treatment strategies may become available.

In addition to the accepted wisdom of the importance of *P. aeruginosa* and CFPEs, others have suggested a role for the Streptococcus Milleri group (SMG) (Sibley *et al.* 2008a). It has been noted in Section 5.1 that almost no bacterial clones from this group were detected from sputum samples at times of stability. In this data using T-RFLP before, during and after CFPEs, no evidence for a role for bacteria from the SMG was found.

8.4.3 Polymicrobial diversity in the CF airways: beyond bacteria

Despite the significant attempts to unravel the complexity of symptoms and microbiology of the CF airway, much remains unclear. Since the inception of this study, a number of key pieces of research have been published, some of which have already been alluded to. Crucially, even greater microbial diversity has been reported. Nagano *et al.* have shown that there are large numbers of diverse fungal species present in the CF airway (Nagano *et al.* 2009). This is interesting from an ecological point of view, as fungi and bacteria are capable of communicating and interacting. Kerr *et al.* showed that a number of *P.*

aeruginosa- derived compounds inhibited growth of *Candida albicans* and *Aspergillus fumigatus in vitro* (Kerr *et al.* 1999). Such a finding is in keeping with clinical observations in CF (Hughes & Kim 1973). In addition, *Candida albicans* has been shown to be capable of producing chemicals that can inhibit the quorum sensing signals in *P. aeruginosa* that are so important fin biofilm production (Cugini *et al.* 2007). In addition there is also increasing evidence notably from a recent observational study (Amin *et al.* 2010) that the persistent presence of *Aspergillus* spp. in CF sputum, without evidence of Allergic Bronchopulmonary Aspergillosis (ABPA), is a strong risk factor for admission to hospital with a relative risk of 1.94. It is worth noting that it is now known that *A. fumigatus* is capable of growing as a biofilm (Loussert *et al.* 2009).

The other work to open up new kingdoms of microbial diversity in CF showed that there may be an enormous diversity of DNA viral communities in the CF airways (Willner *et al.* 2009). Virus have been previously implicated in the pathogenesis of CFPEs (Efthimiou *et al.* 1984; Hiatt *et al.* 1999; Hordvik *et al.* 1989; Wat *et al.* 2008b). In subjects with preceding established airway infection it is possible that the airborne arrival of sufficient quantities of a virus contribute to perturbing the established balance of a bacterial community, thus contributing to the pathogenesis of a CFPE. To date no work presented here is able to confirm of refute such a hypothesis, but it provides an interesting future avenue for investigation. In addition to these potentially negative effects of viruses in the CF airway, there is also the possibility that viruses may be able to play a role in a more positive way through the role of bacteriophages which have been shown to be able to modulate biofilms and influence *P. aeruginosa* phenotypes (Azeredo & Sutherland 2008; Brockhurst, Buckling, & Rainey 2005).

One prominent finding from this work is that bacteria that are typically considered part of the oral microbiota can be permanent and important constituents of the bacterial community in the CF airway. In order to inform the direction of future studies it is of great interest to ascertain the origins of the bacteria that constitute the CF airway bacterial community. The work presented here would add support to the hypothesis that a significant proportion of the CF bacterial community originated in the oropharynx. It seems very likely that micro-aspiration occurs from the oropharynx in both healthy subjects and patients with CF from a young age (Blondeau *et al.* 2009; Ledson *et al.* 1998; Ledson, Tran, & Walshaw 1998a). The healthy subject would be able to withstand small quantities of erosive and infective material, but the same may not be true of subjects with CF. This gives rise to at least two potential areas for future study. The first is the role of reducing the reflux and aspiration of gastric contents, irrespective of its pH. The second is the role of oral health. The teeth are well known to harbour a diverse community of bacteria, including many anaerobes (Filoche, Wong, & Sissons 2009; Gibbons *et al.* 1963). As such both gastric refluxate and oral micro-aspirates may be a source of the bacterial diversity identified in this work.

Another situation where lower respiratory tract infection is a major cause of morbidity and health care cost is in Ventilator Associated Pneumonia (VAP). In VAP, a patient who is immuno-suppressed by the nature of their acute illness, is ventilated using an endotracheal tube (ETT). This ETT may act as a ladder to allow the migration of bacteria from the chronically colonised upper respiratory tract through the usually impregnable “doors” to the LRT that constitute the vocal cords. A number of microbiological interventions to reduce the incidence

of VAP have been subject to large scale RCTs. These include the use of chlorhexidine mouth care around the time of intubation (DeRiso *et al.* 1996; Segers *et al.* 2006), and the use of ETTs impregnated with silver as an antimicrobial agent (Kollef *et al.* 2008). Such studies suggest that it may be worth considering similar strategies for CF patients. As yet, it has not been documented whether subjects with worse oral hygiene have earlier or more rapidly progressive lung disease. One final point to conclude on the connection between the oral cavity and the lower airway bacterial community in CF, is that in mouse models of CF is noted that the CFTR protein has been found to bind and phagocytose *P. aeruginosa* in wild type but not $CFTR^{-/-}$ knockout mice, leading to lower airway colonisation (Coleman *et al.* 2003). This might suggest that the long established and important CF pathogen *P. aeruginosa* may early persistence in the oral cavity as a stepping stone to the lower airways. Indeed there are to date no convincing clinical data to suggest exactly by what route *P. aeruginosa* enters the lung, by inhalation (Wainwright *et al.* 2009), or by oral micro-aspiration (Button *et al.* 1997).

8.4.4 Limitations of Culture Independent Techniques

Culture-independent microbiology techniques offer many potential advantages over traditional selective culture based techniques such as lack of selection bias. As with all methods each has advantages and disadvantages, as outlined in Section 2.3 above (Nocker, Burr, & Camper 2007). For bacterial community profiling and compared to earlier molecular methods such as DNA:DNA re-association kinetics (Torsvik 1980; Torsvik, Goksoyr, & Daae 1990), T-RFLP offers many advantages, such as relatively quick results, relatively high throughput and also some approximate speciation (Liu *et al.* 1997). It also

offers better reproducibility and higher measures of diversity than Length Heterogeneity PCR (Rogers *et al.* 2003). An additional potential concern with T-TFLP as a technique is that the number of copies of the 16S ribosomal RNA gene varies between species, and thus there may be a bias in the apparent relative abundances detected (Farrelly, Rainey, & Stackebrandt 1995).

Due to the limitations of T-RFLP listed above, an additional technique was employed to add specificity to the identification of bacteria. Here, 16S ribosomal RNA clone sequence analysis, considered the 'gold standard' for bacterial identification (Dowd *et al.* 2008) was also carried out. Such analysis was performed on a sample from each subject, from a time distant to treatment for a CFPE. It was not possible to undertake this on more than one sample due to the additional time and cost involved in this technique, but in terms of the objectives of this study, such an approach was deemed to be appropriate. The results are presented in Section 5.1. In summary a median of 7.5 species per subject were detected (IQR 5.75-17.75). In addition 15 species not previously described in CF samples were identified. This is also in keeping with previous work and confirms previously described bacterial diversity in CF respiratory secretions (Bittar *et al.* 2008; Rogers *et al.* 2004). This clone sequence analysis serves a number of purposes in this study. Firstly, it confirms the previous findings of higher bacterial diversity than that detected by culture based methods. Secondly, it allows greater certainty in the speciation of recognised T-RF bands detected by T-RFLP. Finally it allows greater certainty when assigning a species name to a T-RF band. As previously discussed it not possible to ascribe a species name to each T-RF band with absolute certainty, due to between 36,000 to 2 million bacterial species, and 'only' ~900 bases

within the 16S RNA gene. Thus 16S ribosomal gene clone sequence analysis allows more accurate speciation of T-RF bands that cannot be assigned to species by *in silico* analysis of the T-RFLP results.

The results of clone sequence analysis can be used to review the results of T-RFLP. It is noted from Principle Component Analysis of stable samples that a species with a band length of 268 bases appears to be an important in differentiation between frequent and infrequent exacerbators (see Figure 6-13). The source and identity of this band would be of great importance as there is data here to suggest that it may play a significant role in CFPEs. However, the results of bacterial clone sequence analysis fail to provide clues or answers as to the identity of this band, as no species is identified that would give rise to a predicted cut point at this length. Given that it can be seen from Table 5-3 that T-RF 268 constitutes the dominant band in 5 of the 14 subjects and is in the top three species in two others, this is somewhat surprising. It is possible that a new species did indeed become dominant following the start of the study (the time that the clone sequence analysis). Another possibility is that it is derived from a species that was detected by clone sequencing but that does not have a predicted cut in 268 bases, as currently registered online with the BLAST database (see Section 2.3.2). However, it is a possibility that band 268 is in fact derived from another, more dominant, species such as *P. aeruginosa*, as has been shown to be a possibility with band 567, as outlined in Section 2.3.2.1 above. If this is the case, the genetic sequence of the rRNA gene that is the target of T-RFLP may have altered in some way. Significant evolutionary changes in *P. aeruginosa* have been seen to take place in chronic infection of

the CF airway *(Smith et al. 2006a)* favouring decreased virulence and increased persistence.

A large proportion (44%) of the species both detected by T-RFLP and clone sequencing were identified as obligate anaerobes as well as constituents of the normal oral bacterial community. Given that all the samples in this study were spontaneously expectorated samples, this might give rise to a potential criticism. It is a theoretical possibility that the anaerobic bacteria detected are not, in fact, members of the bacterial community in the lungs but merely contaminants of the sputum bolus acquired in its passage through the oropharyngeal cavity. However, such criticisms have been comprehensively dealt with in many ways in previous publications, and with additional evidence in this work. Firstly, paired mouthwash and sputum samples show very poor species concordance, although there was some similarity (Rogers *et al.* 2006). To counter the claim more robustly, others have obtained samples without the risk of oropharyngeal contamination, with samples obtained by fibreoptic bronchoscopy. For the multiple repeated samples required for this study, such invasive sampling methods were not appropriate. Samples obtained in such a way have been subject to anaerobic culture (Tunney *et al.* 2008), and ribosomal gene clone sequence analysis (Bittar *et al.* 2008). In addition, this present study has shown that bacteria from e.g. the genus *Prevotella* are members of the core species in the community of bacteria in the lower respiratory tract, and not merely detected as a result of occasionally poorly prepared sputum samples.

Propidium monoazide

As previously discussed, a new method of preparing the samples prior to T-RFLP became available during the course of the study (Nocker *et al.* 2007). The use of PMA allows the removal of extracellular nucleic acid material, and is able to bind and remove from further analysis the nucleic acid from within non-viable cells. This allowed the T-RFLP process to be refined so that the step of RT-T-TFLP (used to demonstrate the presence of metabolically active, viable bacteria) was no longer required. T-RFLP on samples that had undergone PMA treatment would only amplify nucleic acid from bacterial cells that were intact and viable. All results presented in the molecular CFPE analysis (Chapter 6) were subject to treatment with PMA. All samples in the Periodic Stable Sampling analysis Chapter 5) were analysed by T-RFLP and RT-T-RFLP, without the benefit of PMA treatment.

8.5 Future Directions

The work presented here has revealed a large amount about the complexity of CF pulmonary disease. There are many different directions to take and a number are discussed below.

Clinical Monitoring

The work presented here shows previously unsuspected complexity and day to day variability in symptoms in adults with CF. This findings clearly demonstrate that these components, that constitute a major proportion of the plethora of symptoms that constitute a CFPE (Fuchs *et al.* 1994; Ramsey *et al.* 1999; Rosenfeld *et al.* 2001), are part of a continuum with no clear way to determine a

CFPE. This goes some way to help understand why defining a CFPE has proved so difficult (Marshall 2004).

The data presented here suggests that VASs are a good tool for monitoring changes in individual patients or subjects, but do not allow for easy comparison between patients or subjects. One suggestion for the future is that VASs may provide a useful tool in the clinical setting to allow different clinicians to monitor symptoms changes between clinic visits in a more objective manner than is currently possible. For example it would allow a clinician to attach a numerical value to the answer to the oft asked question "How are you today compared to last visit?". Providing an objective measure of a subjective phenomena is likely to become more important as the number of clinicians involved in CF care continue to rise in line with the expanding adult CF population (Dodge *et al.* 2006).

Sputum Inflammatory Mediators

Sputum inflammatory mediators are a challenging area. The findings presented here show that sputum ECP correlate to indices of bacterial community structure. The findings suggest that the more even the community, the greater the amount of ECP. This finding is in keeping with others within this study, notably that there are fewer species detected, with a less even community structure during treatment for a CFPE, than in the preceding three weeks. However, the biochemical link between ECP, derived from the host inflammatory cells, and these bacterial community structure indices is not clear.

Microbiology

The somewhat unexpected observation that treatment for CFPEs results in a dramatic fall in the proportions of anaerobic bacteria, but no significant change in the proportion of *P. aeruginosa* leads to a number of further possible avenues for investigation. Given the increasing predicted life expectancy of people with CF (Dodge *et al.* 2006) side effects from treatment are becoming increasingly important and serious for patients (Al-Aloul *et al.* 2005; Bertenshaw *et al.* 2007). Thus strategies aimed at reducing the reliance on toxic antimicrobial therapy are needed. Oxygen therapy targeting anaerobes such as *Prevotella* (Takumi *et al.* 2008) may be able to play such a role.

New Insights in to Mechanisms of Action for Established Therapies

The findings that are presented here suggest additional explanations to the accepted mechanisms of action for proven therapies for CF. Hypertonic saline, for example, has been shown to be beneficial in large RCTs in CF (Elkins *et al.* 2006). It is hypothesised to work by correcting the defective dehydration in the airway surface liquid in CF, thus allowing more effective mucociliary clearance (Boucher 2007). Thus may indeed be true, but the evidence presented here suggests that there is another possibility: that hypertonic saline is influencing the microbial community. This may be either by reducing the passage of bacteria from upper to lower airway akin to mouthwash in VAP (Koeman *et al.* 2006), or by altering the microbial habitat such that the microbial residents are changed in composition or activity.

There are at least two other important proven therapies in CF that may exert an effect though influencing the microbial community. The first is Azithromycin, which has been shown to have considerable beneficial effects in long term therapy, in reducing exacerbations, and increasing lung function and weight (Jaffe *et al.* 1998; Saiman *et al.* 2003; Wolter *et al.* 2002). Putative mechanisms of action include as an anti-inflammatory agent (Ianaro *et al.* 2000), as a quorum sensing blocker (Hoffmann *et al.* 2007), and as an anti-biofilm agent (Gillis & Iglewski 2004) among others. Which ever of these are involved, it would seem likely that previous studies of the effects of azithromycin on airway microbiology using culture dependent techniques (Saiman *et al.* 2003; Wolter *et al.* 2002) would have been insensitive to the types of changes seen in this work.

Secondly, Dornase alpha is commercially available DNAse that has been shown to be beneficial in CF (Fuchs *et al.* 1994). It is thought to work by thinning what is presumed to be hyper viscous sputum, allowing more efficient expectoration and less damage to the airways as a result of the inflammatory cytokine milieu that is present in the sputum. In addition to this Dornase alpha may have an ecological impact on the bacterial community. It has been shown that a more viscous medium has been shown to encourage co-operation between strains of *P. aeruginosa* (Kummerli *et al.* 2009) and promote biofilm formation (Matsui *et al.* 2006). Thus, by thinning the viscous lower airway secretions, treatment with Dornase alpha may alter the microbial community in the lower airways in CF in a beneficial manner.

A more thorough study of the ecological effects of such treatments *in vivo* would allow greater insights in to mechanisms and open up new avenues of other such therapies.

A Possible Connection between the Upper and Lower Airway in CF

As discussed in the preceding section, the work presented here suggests there may be bacterial colonisation from the oropharynx to the lower airway in CF. There is good evidence that bacterial colonisation of the LRT occurs early in CF, perhaps shortly after birth, and is associated with inflammation (Armstrong *et al.* 1997; Armstrong *et al.* 1995; Armstrong *et al.* 2005). This early stage of CF pulmonary disease is when there is the most potential to limit future damage, and thus reduce morbidity and mortality. Before embarking on a RCT of any intervention to improve oral hygiene with the proposed outcome of reducing bacterial migration to the lower airways, it would be useful to demonstrate a link between poor oral hygiene, either by dental caries measures (Macnab *et al.* 2008), or by assessment of adherence to standard dental hygiene (Narang *et al.* 2003) (brushing teeth, visits to the dentist etc.). It might be expected that those more dental caries, and those with poorer adherence to standard dental hygiene techniques, would have worse pulmonary health. If such a study were to add additional evidence to the hypothesis that oral bacteria contribute to the early infection and subsequent lung damage in CF, then a large scale RCT would be warranted.

One observation that does not have a satisfactory explanation is the marked temporal variation in bacterial community composition and structure seen in some subject, particularly subjects 05 and 09. These are two of the three subjects with the most severe (obstructive) pulmonary disease. As such this offers one possible explanation. As lung disease severity progresses the total amount of ventilated lung decreases, thus increasing the proportion of the lung that is not ventilated. These under ventilated portions of the lung may provide

heterogeneous habitats that may harbour bacterial communities that differ from the communities in other parts of the lung. Different communities may in fact be responsible, in part, for both the heterogeneous damage that is seen in advance CF lung disease (de Jong & Tiddens 2007), and the heterogeneous "hot spots" of inflammation seen in CF lungs during CFPEs (Klein *et al.* 2009). To adequately investigate this idea of heterogeneous communities in different parts of the CF lung further would require more study. Fibreoptic bronchoscopy with BAL would be one means of sampling, but may be sub-optimal due to failure of saline to remove adherent biofilms in the very distal small airway that are the primary area of infection in CF. Additionally there would be high clinical risk due to the multiple areas that would need to be washed out. A more satisfactory study would involve sampling the whole lungs, *ex vivo*, at the time of lung transplantation. Such a solution would also help add strong support to the body of evidence showing that of oral contamination is not a suitable explanation for the high numbers of anaerobic bacteria found in respiratory secretions in CF (Bittar *et al.* 2008; Rogers *et al.* 2006; Tunney *et al.* 2008; Worlitzsch *et al.* 2009).

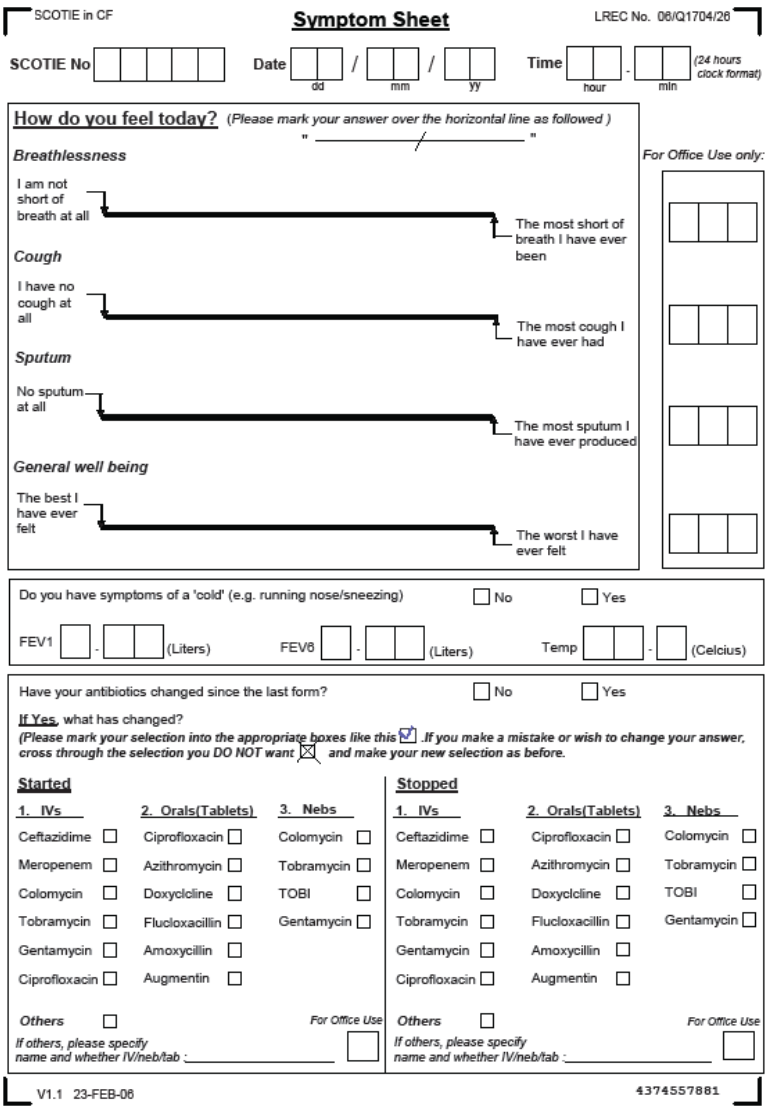
8.6 Summary

The extended body of work presented here covers a wide range of diverse areas in CF pulmonary disease. It has revealed new complexities in symptoms, and cast doubt on current paradigms in understanding what constitutes a clinical diagnosis of a CF pulmonary exacerbation. The finding of fluctuations in symptoms that mimic clinician defined CFPEs suggests that there is an unaddressed burden of disease in CF adults.

The culture independent molecular techniques results have revealed that *Prevotella sp* and other anaerobes may have a crucial role in interacting with *P. aeruginosa* around the time of CFPE. This in turn opens up new avenues for future research, and may influence current medical practise.

Chapter 9 - Appendices

9.1 Clinical Monitoring Form



SCOTIE in CF

Symptom Sheet

LREC No. 06/Q1704/26

SCOTIE No ☐☐☐☐☐ Date ☐☐ / ☐☐ / ☐☐ (dd / mm / yy) Time ☐☐ - ☐☐ (hour - min) (24 hours clock format)

How do you feel today? (Please mark your answer over the horizontal line as followed)

" ——/—— "

For Office Use only:

Breathlessness

I am not short of breath at all — The most short of breath I have ever been

Cough

I have no cough at all — The most cough I have ever had

Sputum

No sputum at all — The most sputum I have ever produced

General well being

The best I have ever felt — The worst I have ever felt

Do you have symptoms of a 'cold' (e.g. running nose/sneezing) ☐ No ☐ Yes

FEV1 ☐ . ☐☐ (Liters) FEV6 ☐ . ☐☐ (Liters) Temp ☐☐ . ☐ (Celcius)

Have your antibiotics changed since the last form? ☐ No ☐ Yes

If Yes, what has changed?

(Please mark your selection into the appropriate boxes like this ☑ .If you make a mistake or wish to change your answer, cross through the selection you DO NOT want ☒ and make your new selection as before.

Started			**Stopped**		
1. IVs	**2. Orals(Tablets)**	**3. Nebs**	**1. IVs**	**2. Orals(Tablets)**	**3. Nebs**
Ceftazidime ☐	Ciprofloxacin ☐	Colomycin ☐	Ceftazidime ☐	Ciprofloxacin ☐	Colomycin ☐
Meropenem ☐	Azithromycin ☐	Tobramycin ☐	Meropenem ☐	Azithromycin ☐	Tobramycin ☐
Colomycin ☐	Doxyclcline ☐	TOBI ☐	Colomycin ☐	Doxyclcline ☐	TOBI ☐
Tobramycin ☐	Flucloxacillin ☐	Gentamycin ☐	Tobramycin ☐	Flucloxacillin ☐	Gentamycin ☐
Gentamycin ☐	Amoxycillin ☐		Gentamycin ☐	Amoxycillin ☐	
Ciprofloxacin ☐	Augmentin ☐		Ciprofloxacin ☐	Augmentin ☐	
Others ☐		***For Office Use*** ☐	***Others*** ☐		***For Office Use*** ☐
If others, please specify name and whether IV/neb/tab : _____			***If others, please specify name and whether IV/neb/tab :*** _____		

V1.1 23-FEB-06 4374557881

Figure 9-1 Clinical Monitoring Form

9.2 Histograms for Clinical Parameters

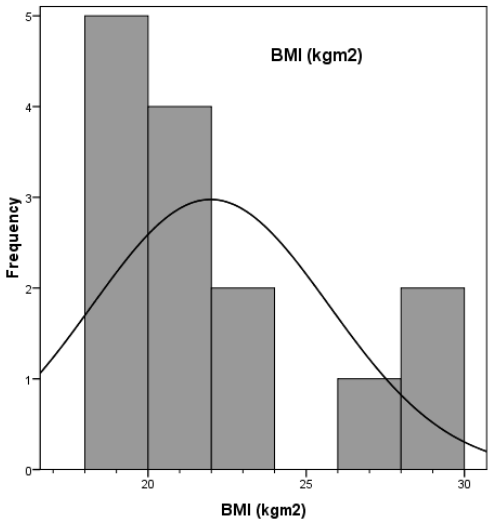


Figure 9-2 Distribution of FEV1

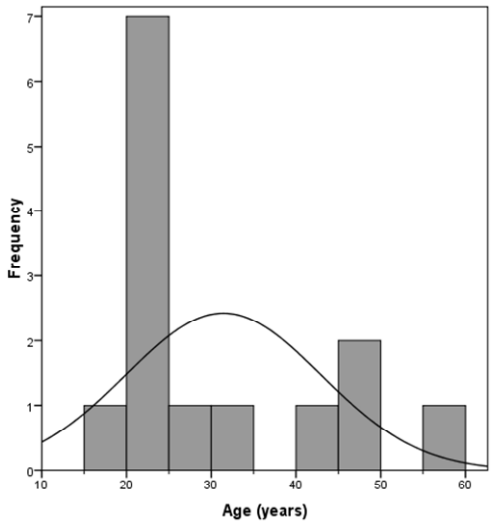


Figure 9-3 Distribution of Age

9.3 Box and Whisker Plot for FEV6 Values for All Subjects

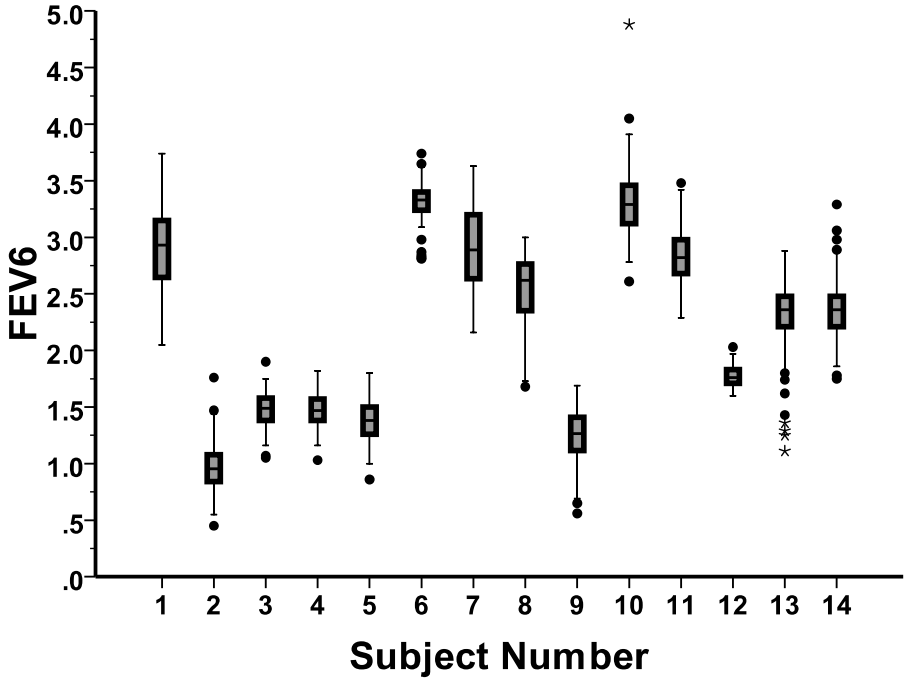


Figure 9-4 FEV6 box and whisker plot

9.3.1 Distribution of Spirometric Values

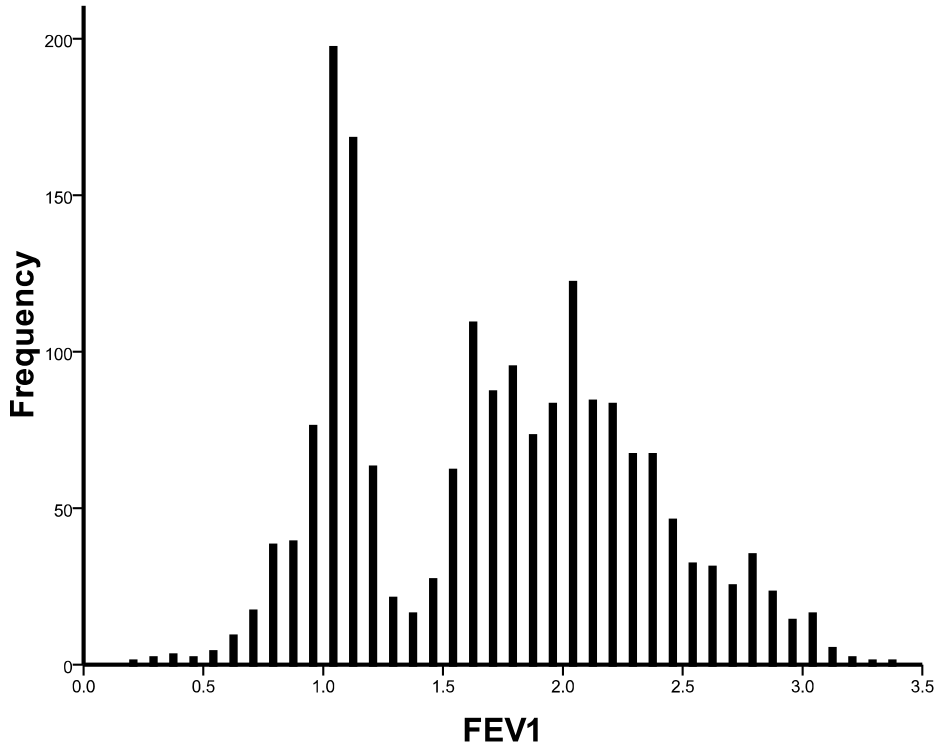
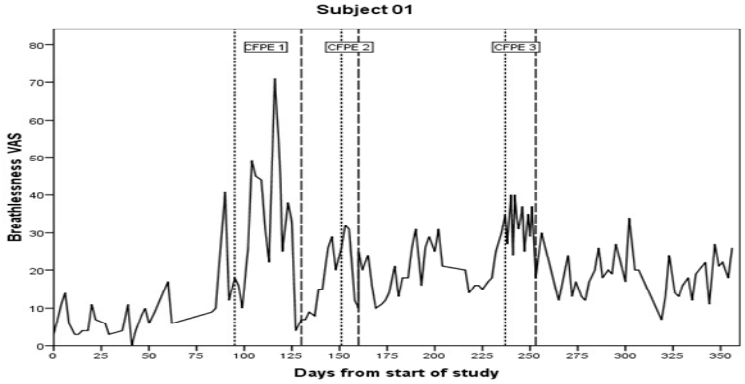
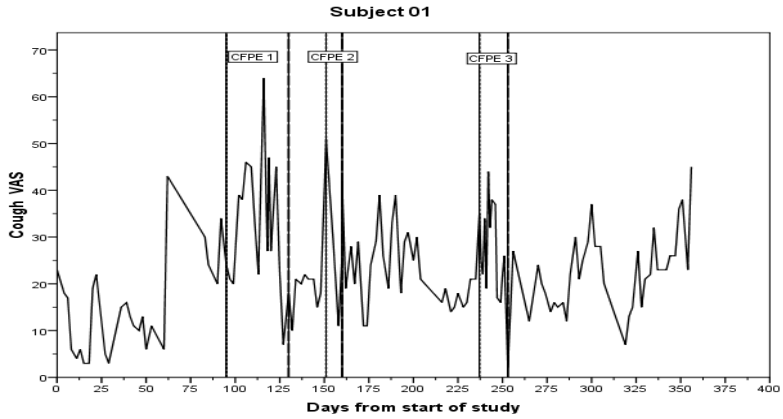


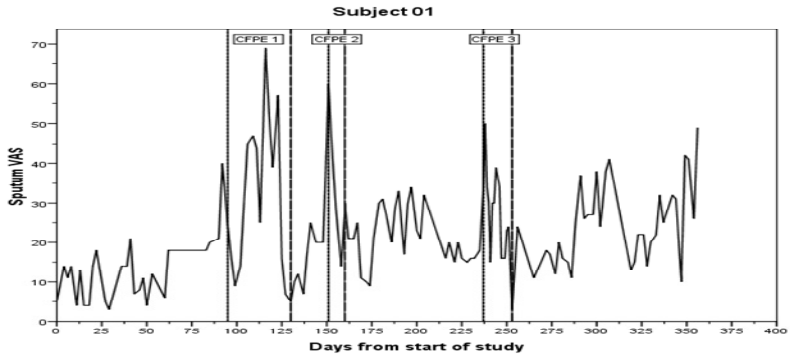
Figure 9-5 Histogram of FEV1 values for all subjects, showing bi-modal distribution.

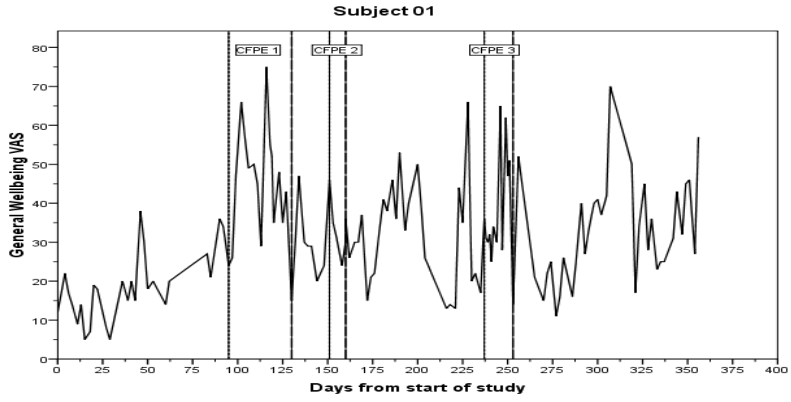
9.4 Individual Response Profile Plots

9.4.1 Subject 01

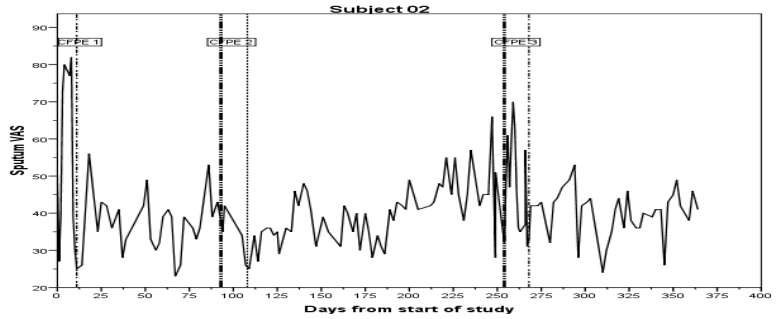


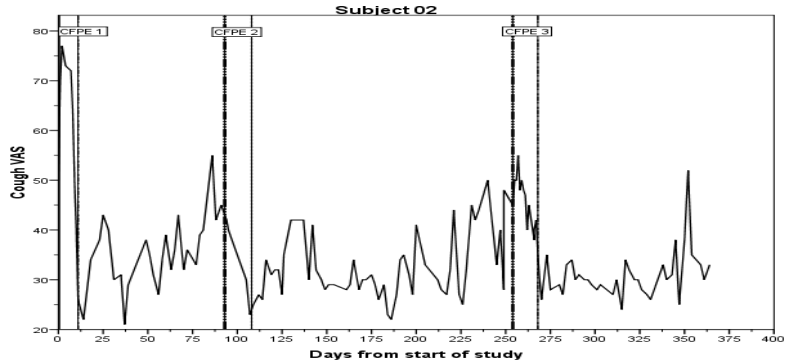


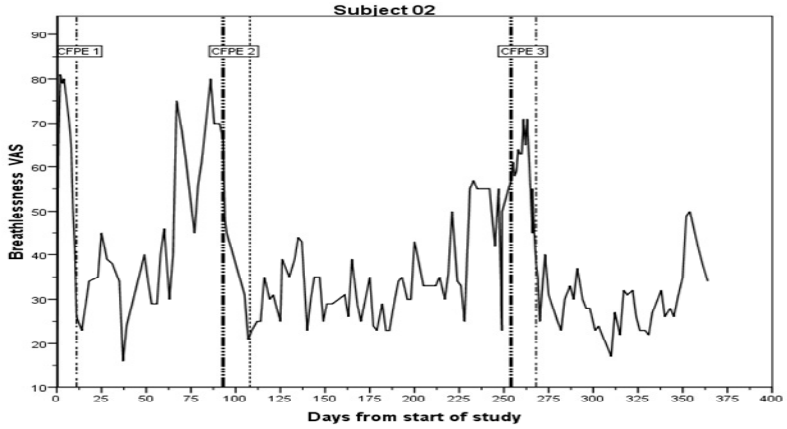


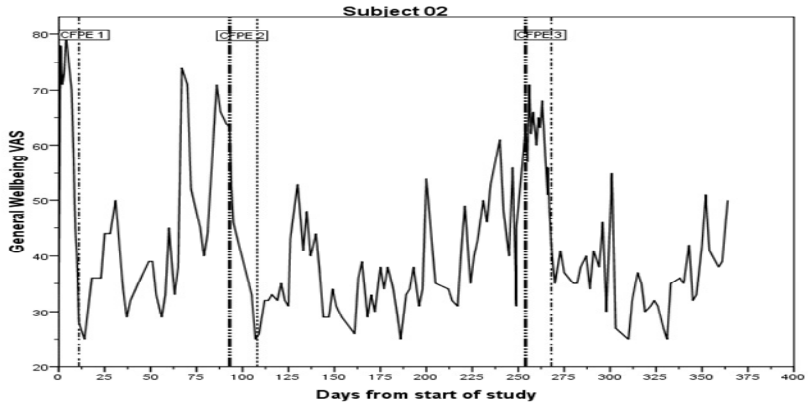


9.4.2 Subject 02









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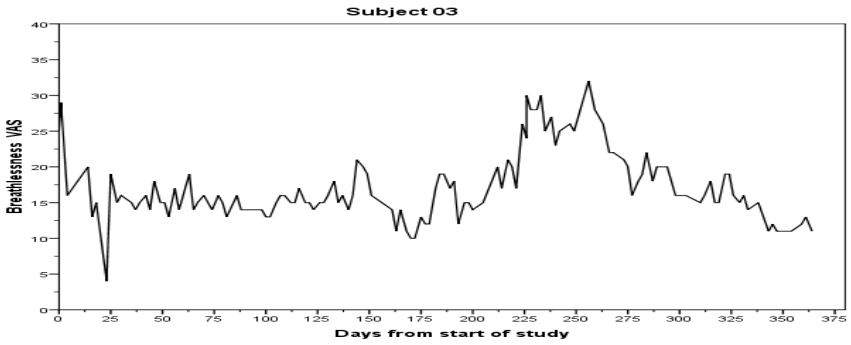

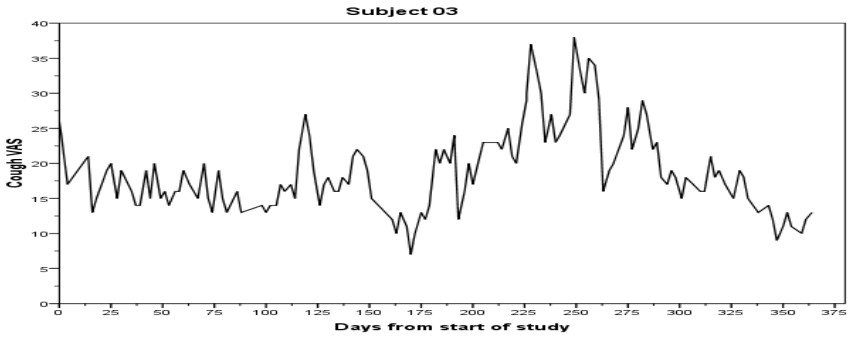

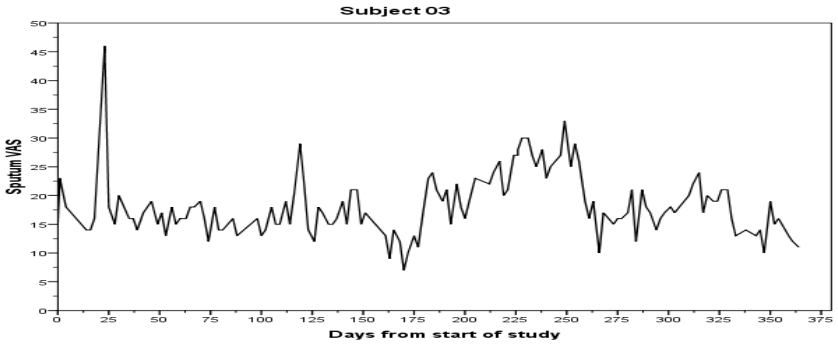

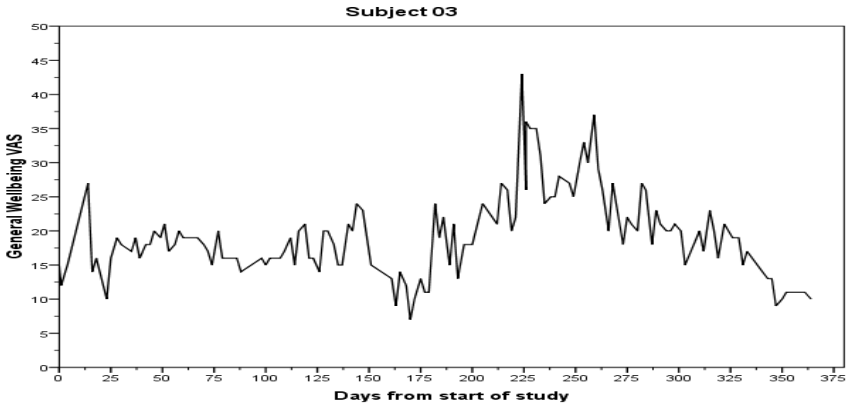

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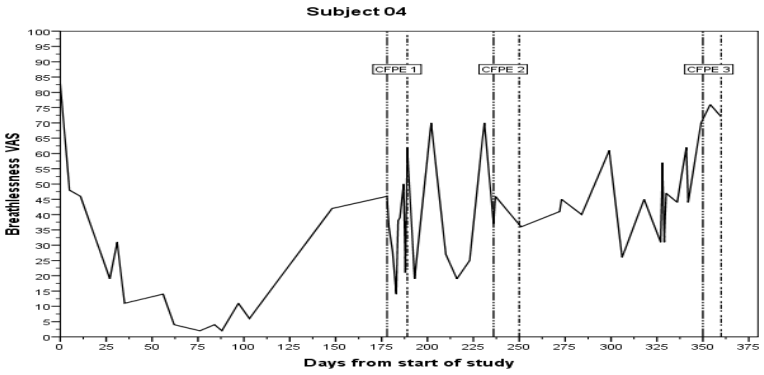

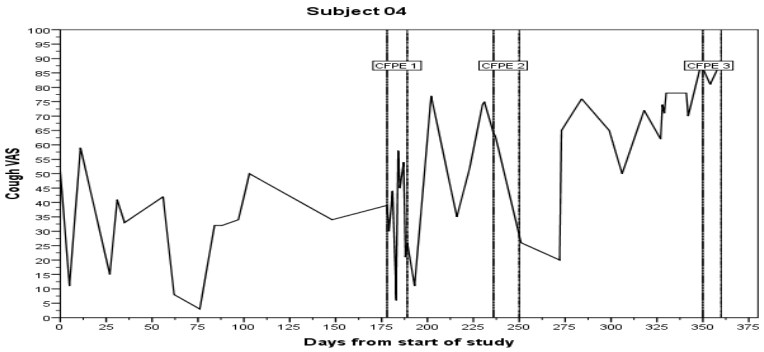

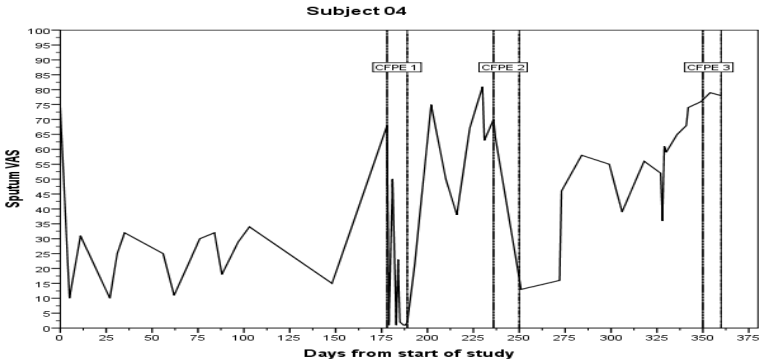

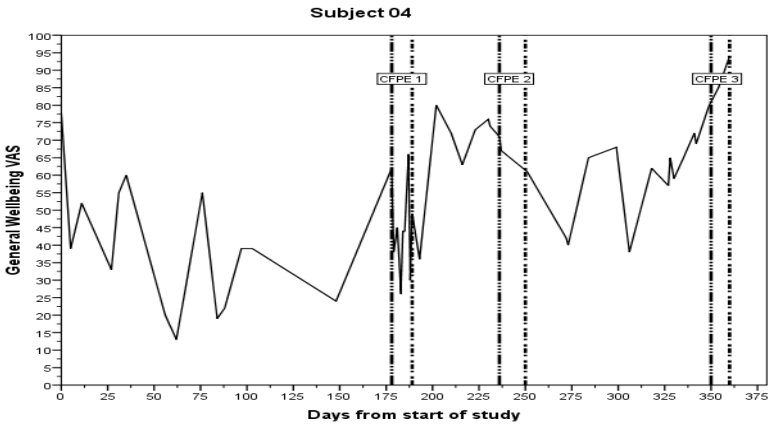

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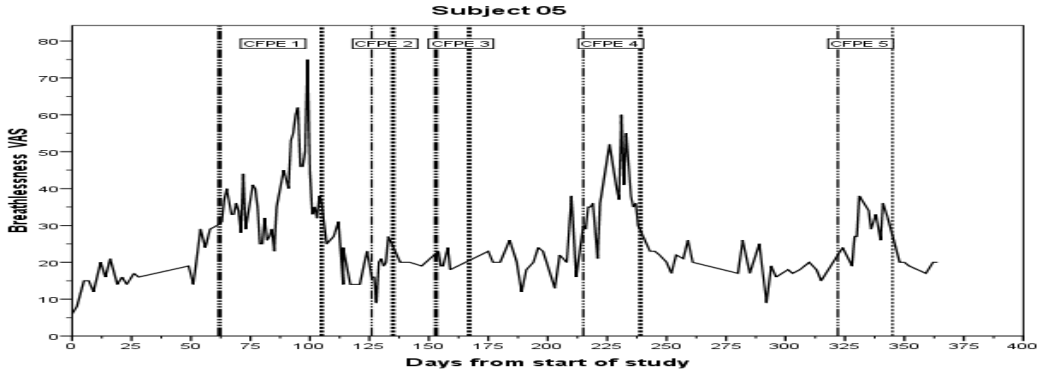

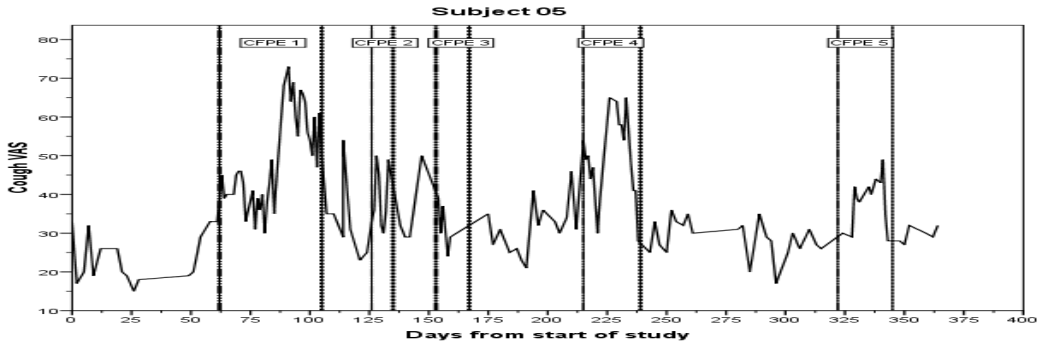

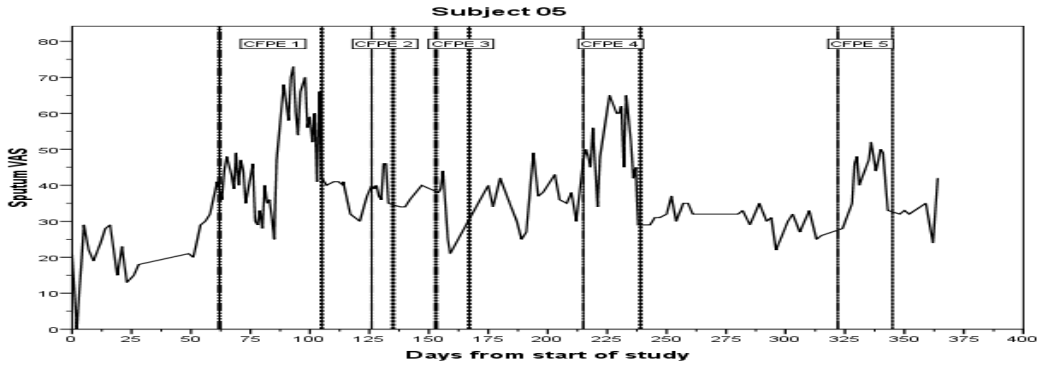

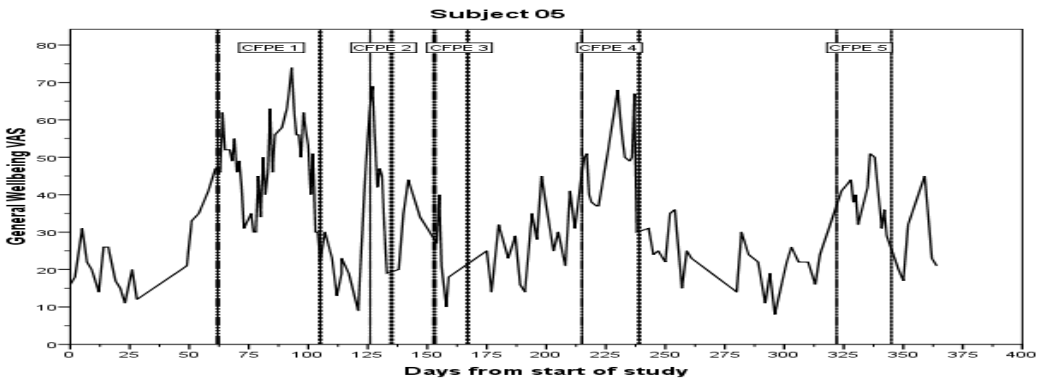

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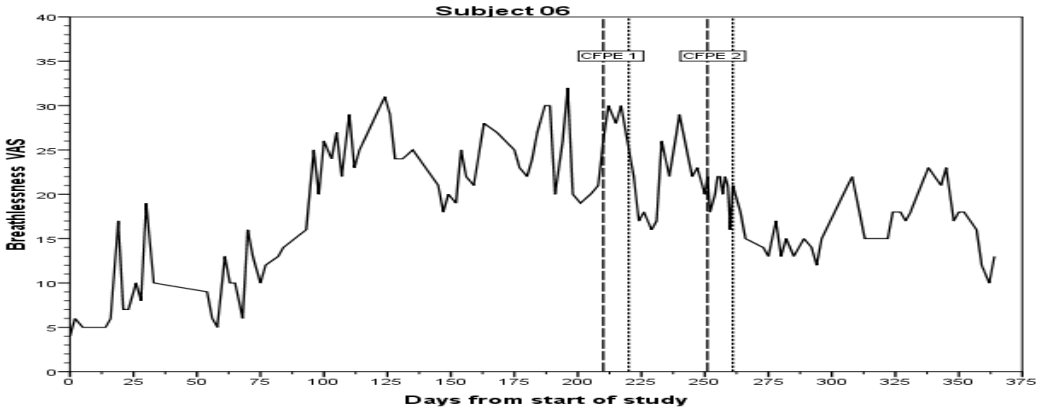

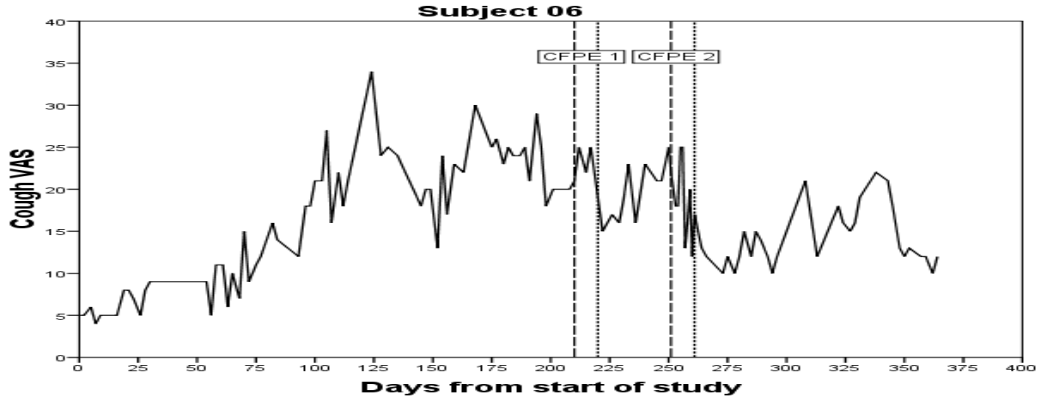

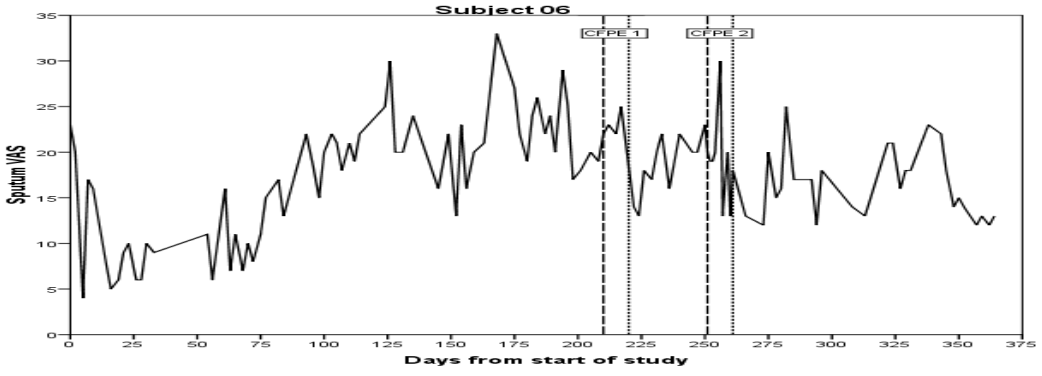

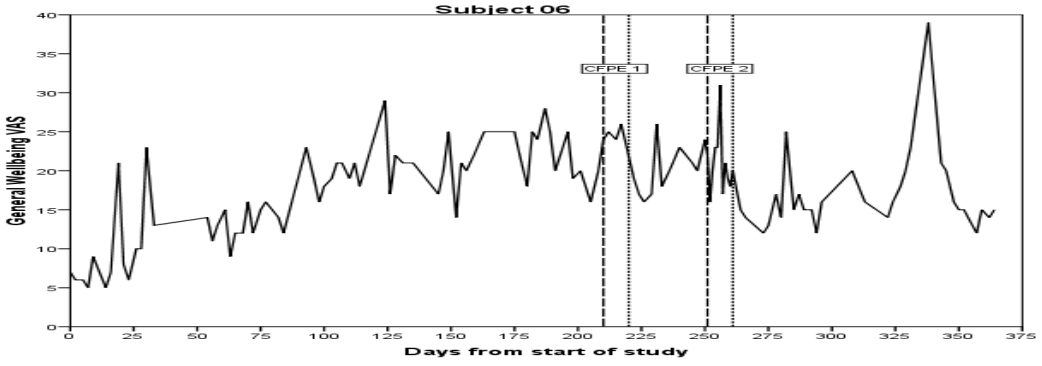

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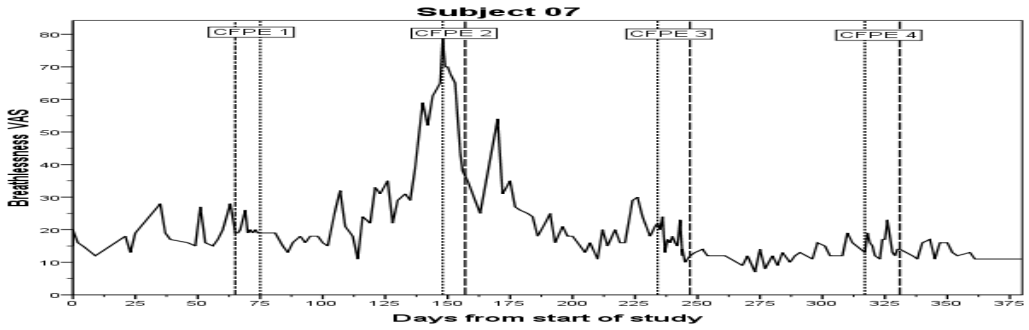

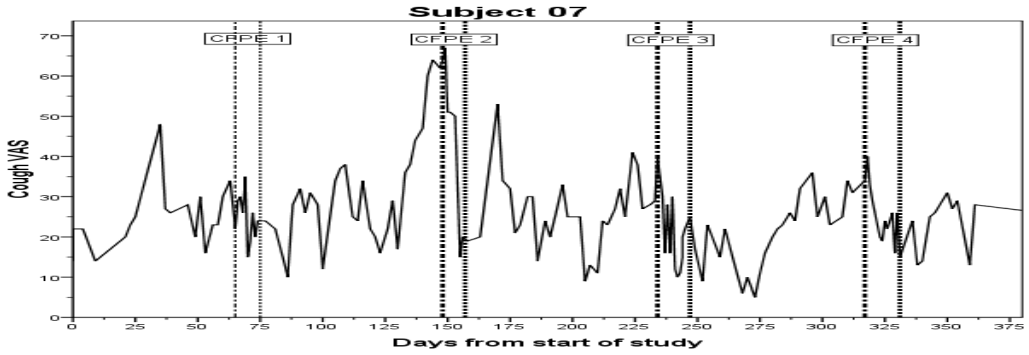

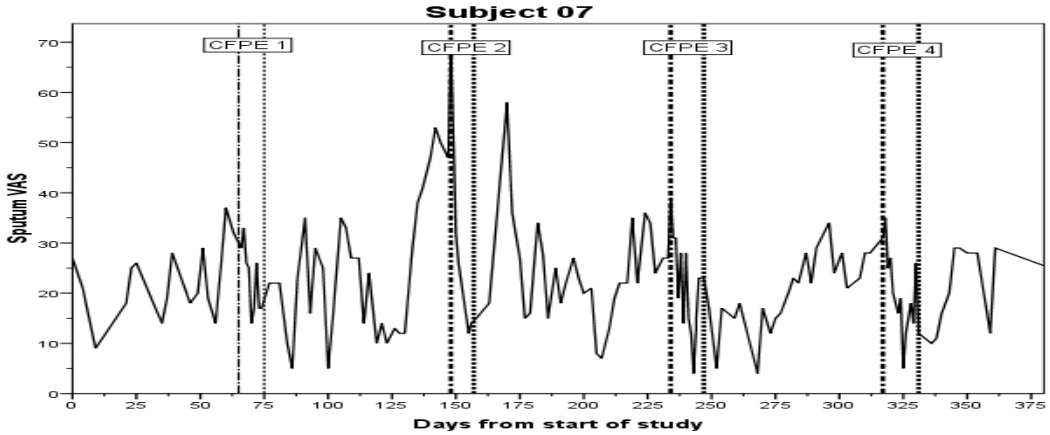

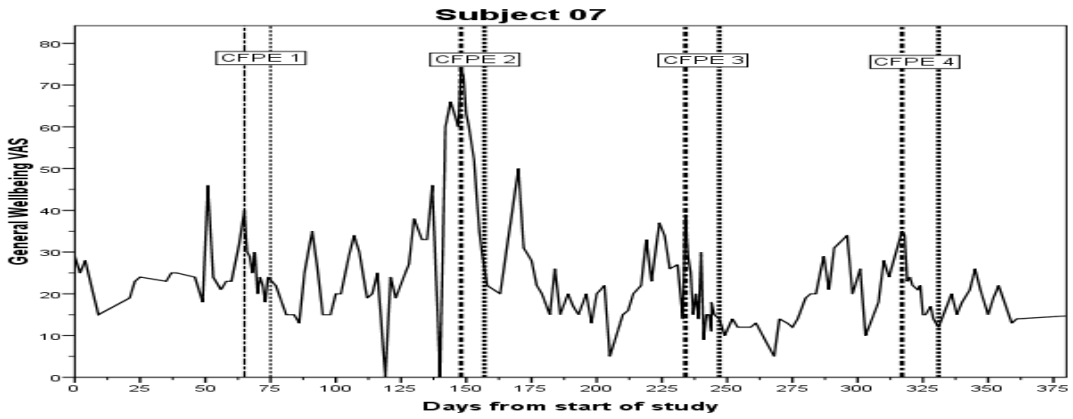

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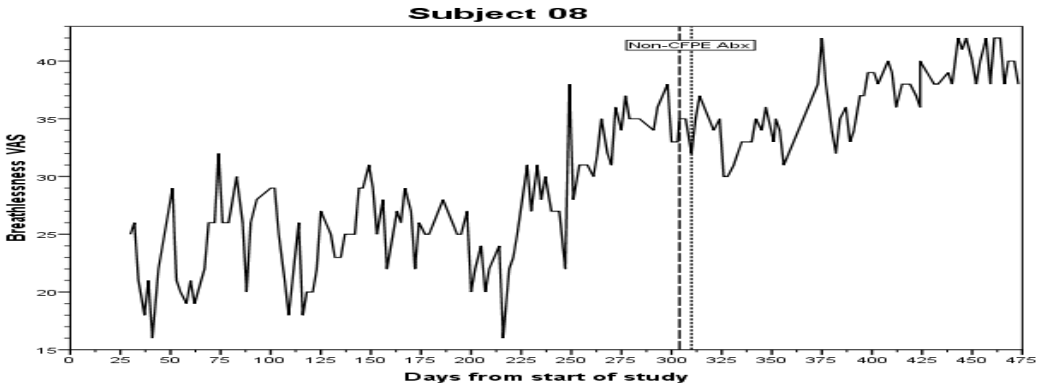
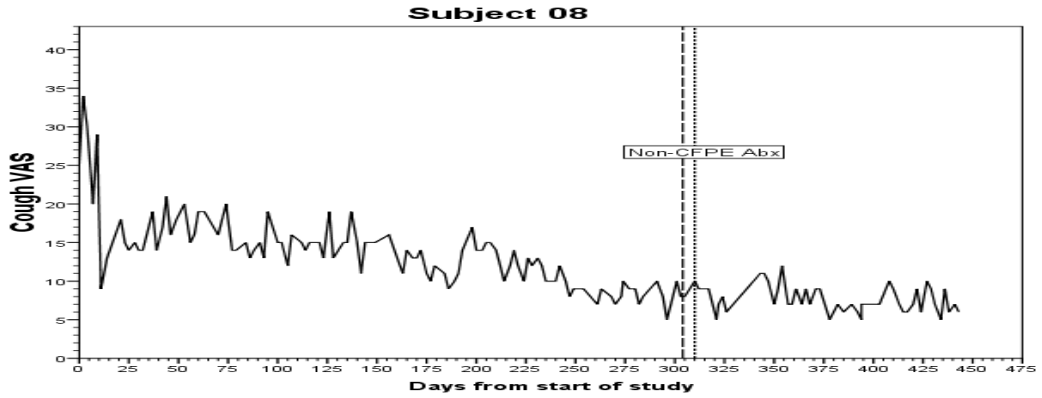
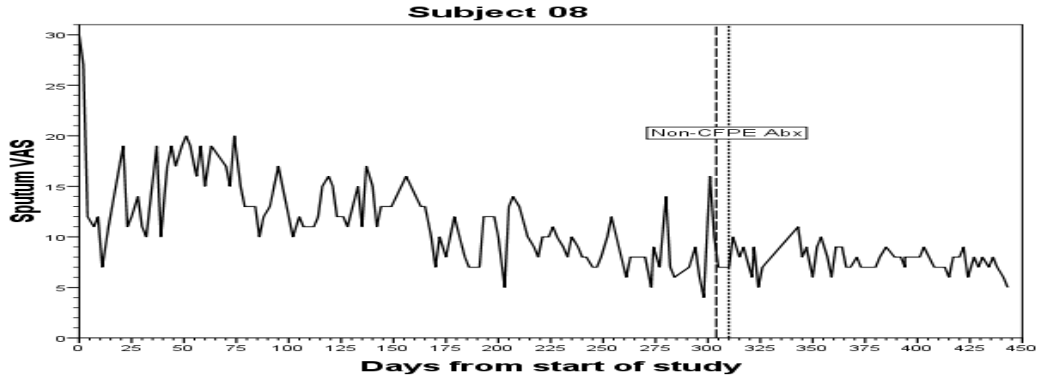
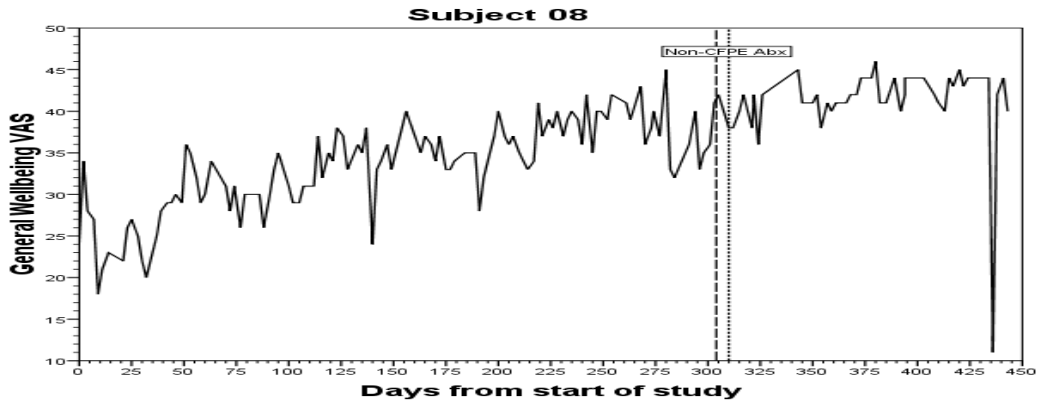
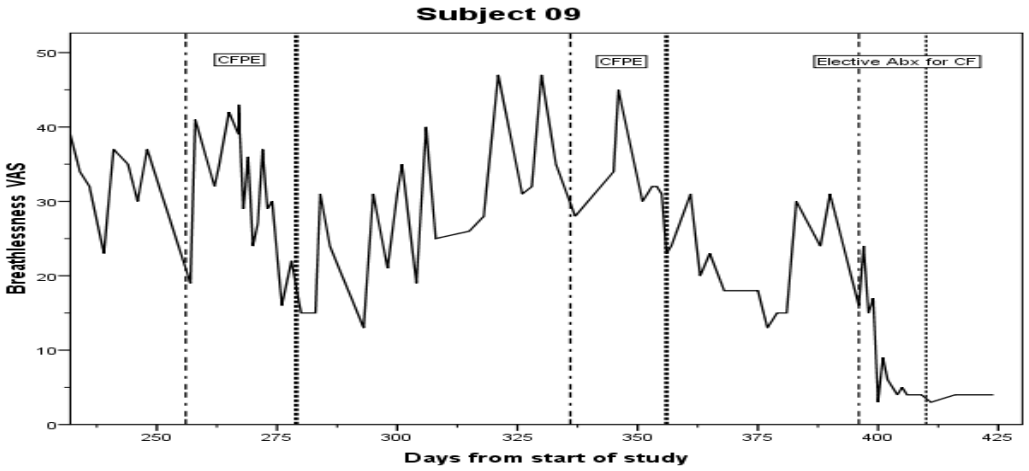


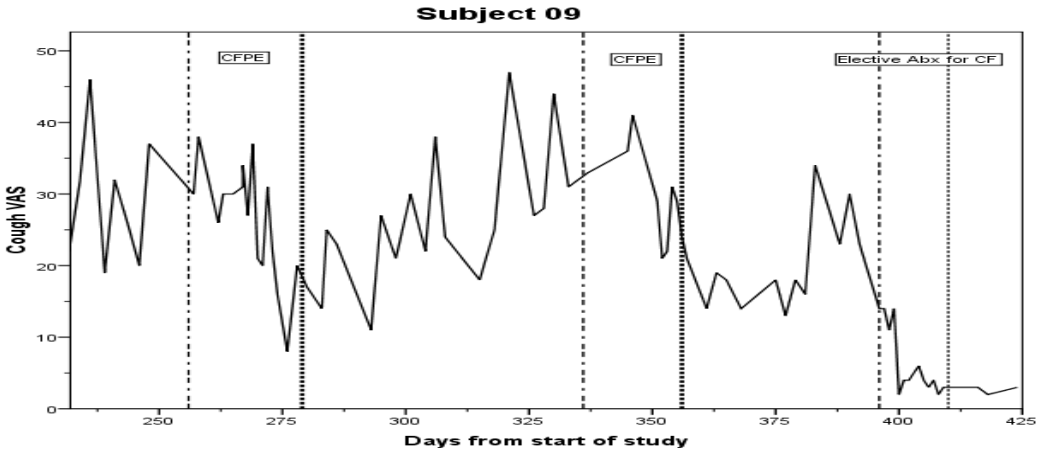


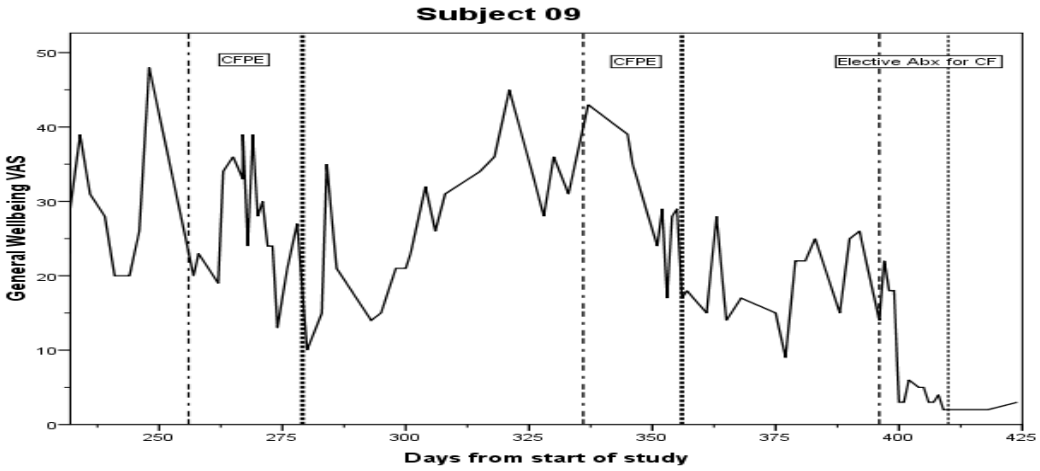




9.4.9 Subject 09







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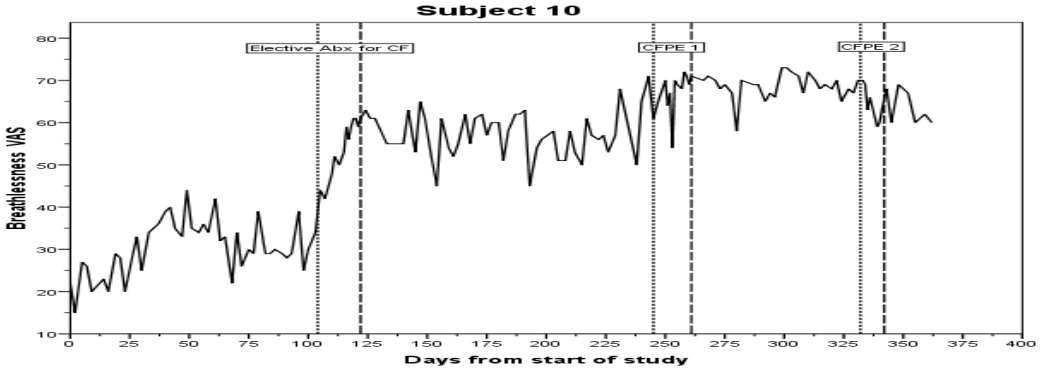
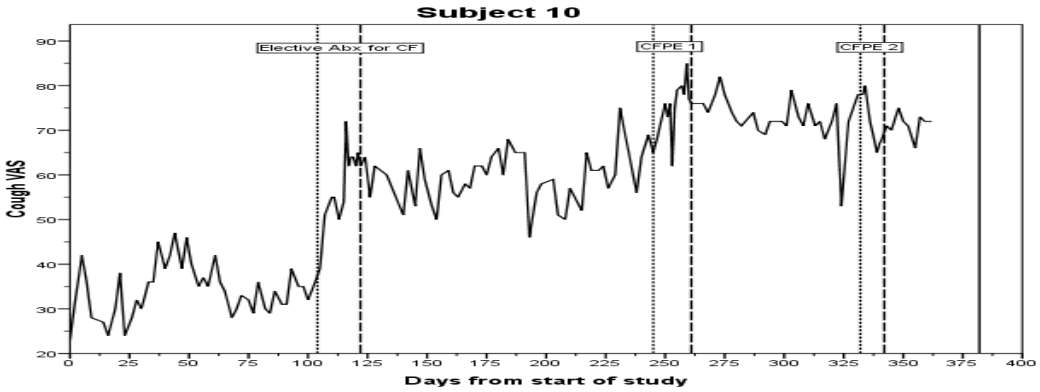
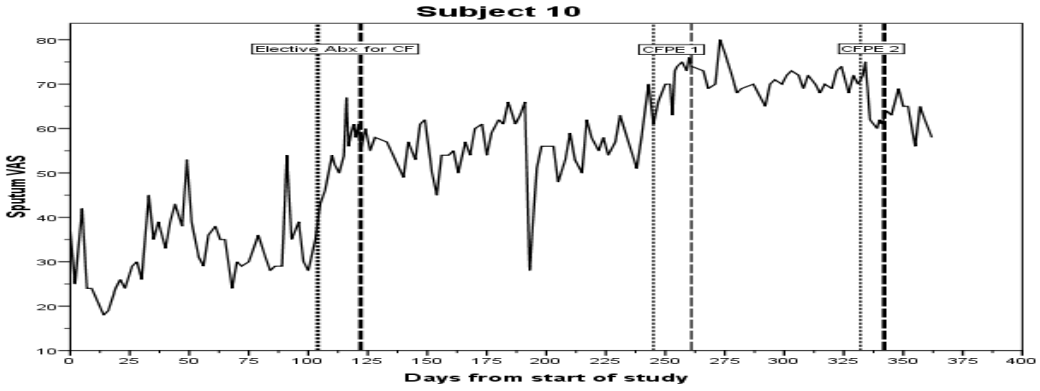
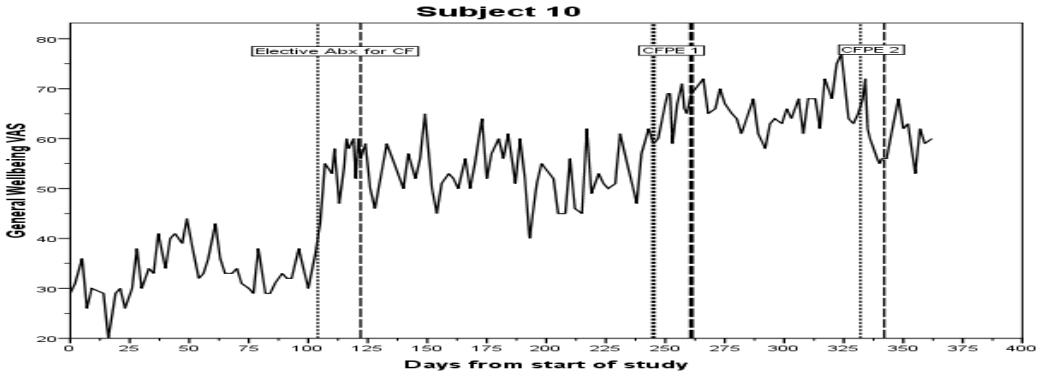
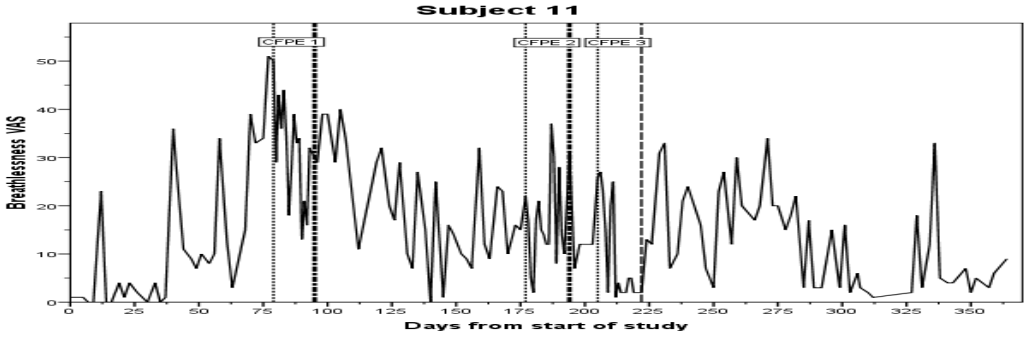


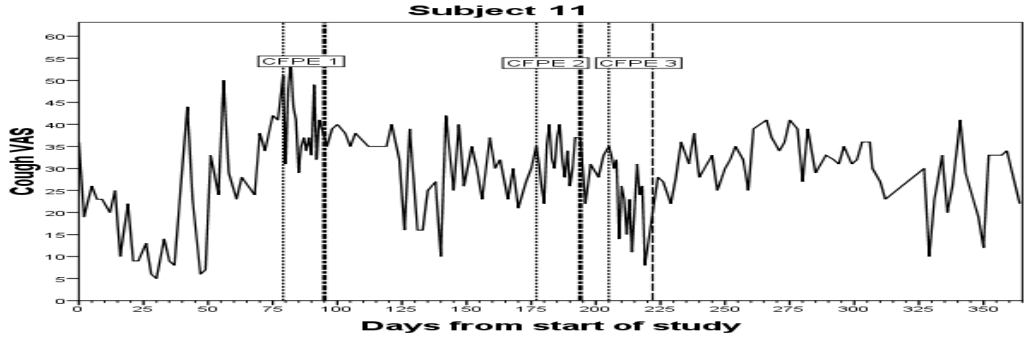


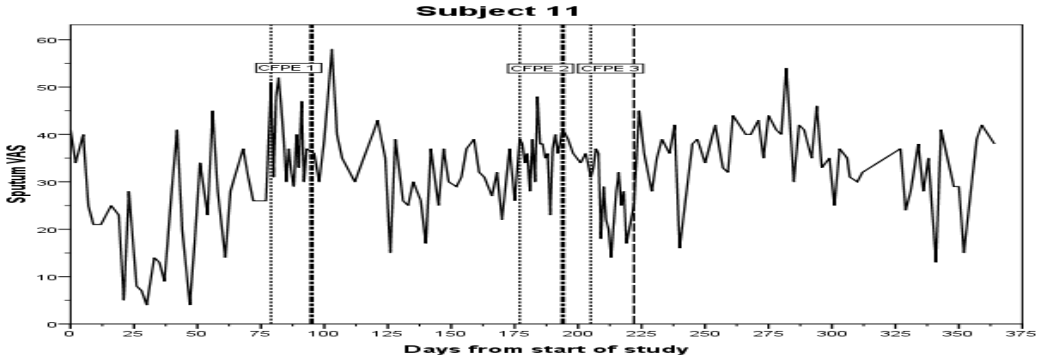


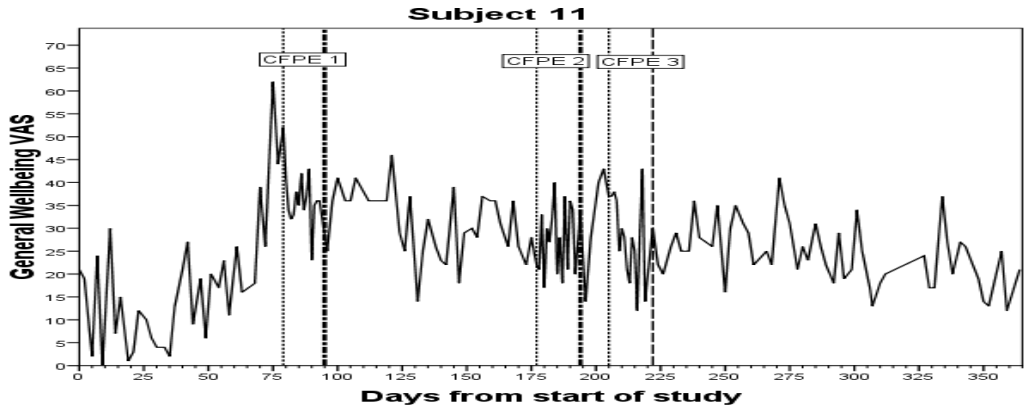


9.4.11 Subject 11

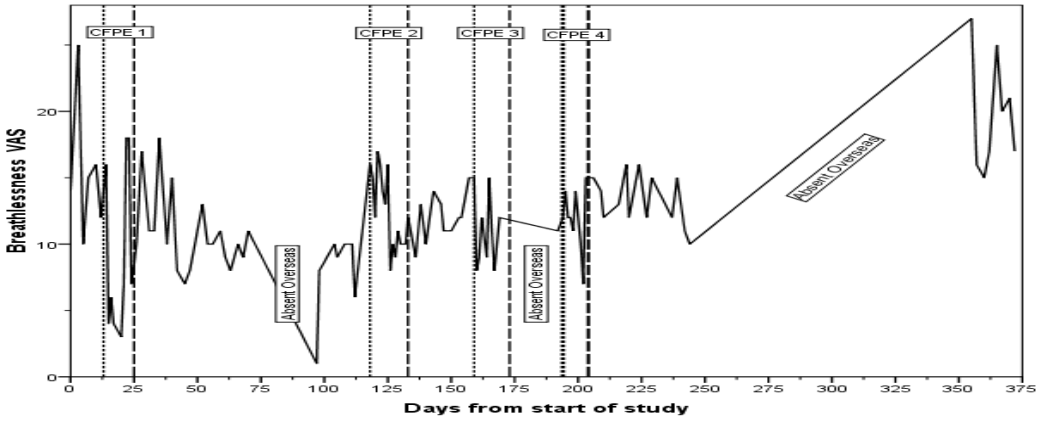


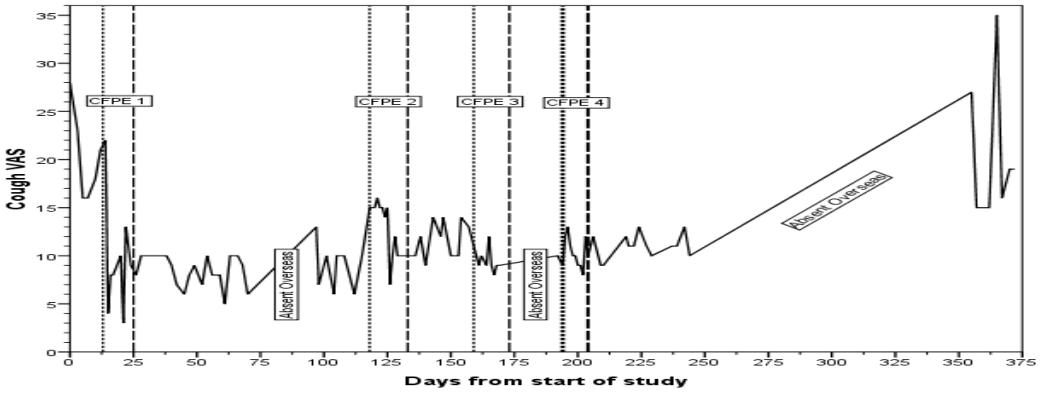


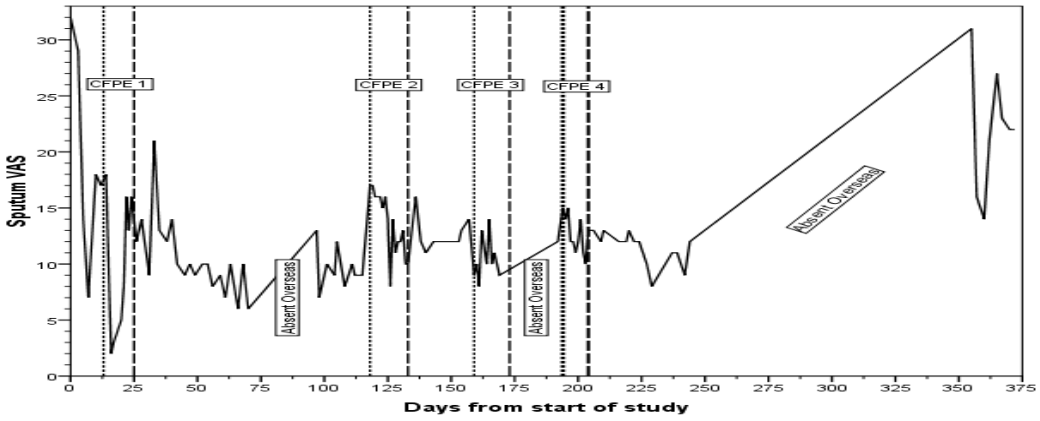


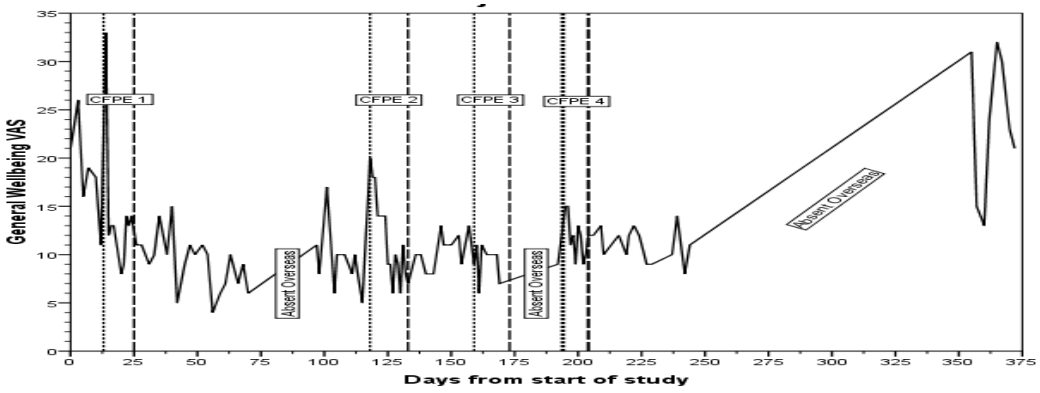


9.4.12 Subject 12









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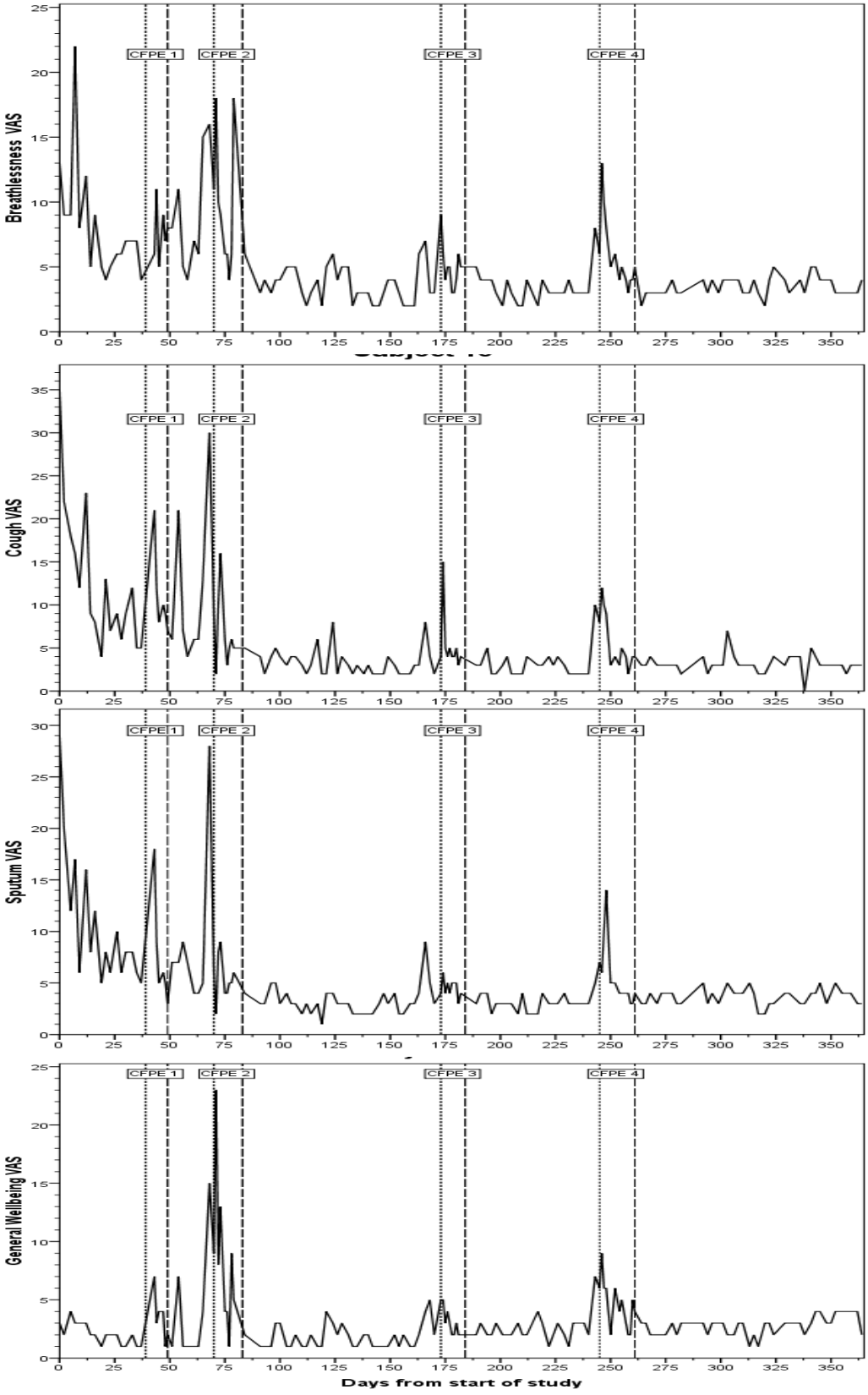
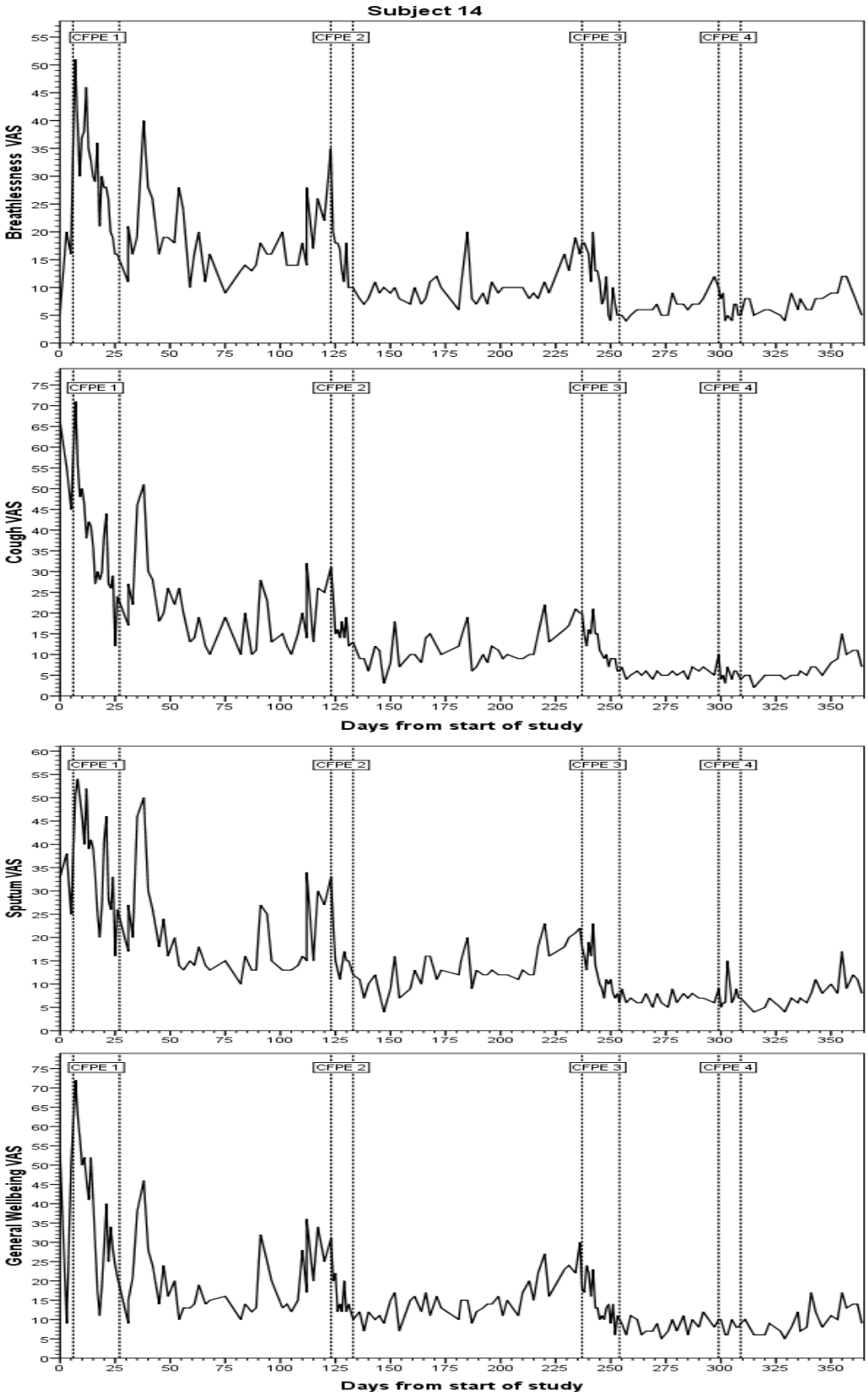


9.4.14 Subject 14



9.5 Bacterial Clone Sequence Results

Family	Genus & Species id	Aerobe/Anaerobe	Oral
Actinomycetaceae	*Actinomyces graevenitzii* (98%)	Ae	O
	Actinomyces lingnae (98%)	Ae	O
	Actinomyces naeslundii	Ae	O
	Actinomyces naeslundii/viscosus (98%)	Ae	O
Micrococcineae	*Rothia dentocariosa* (97%)	Ae	O
	Rothia mucilaginosa	Ae	O
Bacteroidaceae	*Bacteroides acidofaciens* (88%)	An	
	Bacteroides eggerthii (88%)	An	
	Bacteroides uniformis (87%)	An	
	Bacteroides uniformis (88%)	An	
	Bacteroides viscericola (86%)	An	
Porphyromonadaceae	*Porphyromonas catoniae* (92%)	An	O
	Tannerella forsythensis (94%)	An	O
Prevotellaceae	*Prevotella loescheii* (98%)	An	O
	Prevotella melaninogenica	An	O
	Prevotella nanceiensis	An	
	Prevotella nigrescens	An	O
	Prevotella oris (98%)	An	O
	Prevotella oulora	An	O
	Prevotella pallens	An	O
	Prevotella pleuritidis	An	
	Prevotella salivae	An	O
	Prevotella tannerae	An	O
	Prevotella veroralis	An	O
Rikenellaceae	*Alistipes shahii* (82%)	An	O
Flavobacteriaceae	*Capnocytophaga gingivalis*	Ae	O
	Capnocytophaga granulosa (98%)	Ae	O
	Capnocytophaga leadbetteri (98%)	Ae	O
	Capnocytophaga sputigena	Ae	O
Staphylococcaceae	*Gemella haemolysans*	Ae	O
	Staphylococcus aureus	Ae	
Aerococcaceae	*Abiotrophia defectiva*	Ae	O
Carnobacteriaceae	*Granulicatella adiacens*	Ae	O
	Granulicatella para-adiacens	Ae	O
Streptococcaceae	*Streptococcus infantis*	Ae	O
	Streptococcus intermedius/constellatus	Ae	O
	Streptococcus mitis	Ae	O
	Streptococcus mitis/pneumoniae	Ae	O
	Streptococcus oralis	Ae	O
	Streptococcus parasanguis	Ae	O
	Streptococcus parasanguis/oralis/mitis	Ae	O
	Streptococcus pneumoniae	Ae	
	Streptococcus salivarius	Ae	O
	Strep. salivarius/vestibularis/thermophilus	Ae	O
	Streptococcus sanguinis	Ae	O
Clostridiaceae	*Clostridium metallolevans* (98%)	Ae	

Family	Genus & Species id	Aerobe/Anaerobe	Oral
Eubacteriaceae	*Mogibacterium neglectum*	Ae	O
Lachnospiraceae	*Catonella morbi* (98%)	An	O
	Dorea longicatena	An	
	Eubacterium spp. 'Smarlab BioMol-2301166'	An	
	Eubacterium spp. 'Smarlab BioMol-2301231'	An	
	Oribacterium sinus (98%)	An	
	Butyrate-producer SR1/1 (97%)	An	
Veillonellaceae	*Veillonella atypica*	An	O
	Veillonella dispar	An	O
	Veillonella parvula	An	O
	Veillonella ratti (96%)	An	O
	Dialister invisus	An	O
	Megasphaera micronuciformis	An	O
	Selenomonas infelix	An	O
Fusobacteriaceae	*Fusobacterium naviforme/nucleatum*	An	O
	Fusobacterium nucleatum	An	O
	Leptotrichia trevisanii (97%)	An	O
Alcaligenaceae	*Achromobacter xylosoxidans/insolitus*	Ae	
Neisseriales	*Eikenella corrodens*	Ae	O
	Neisseria cinerea	Ae	O
	Neisseria elongata	Ae	O
	Neisseria flava/sicca/mucosa/pharyngis	Ae	O
	Neisseria flavescens (98%)	Ae	O
	Neisseria meningitidis (95%)	Ae	O
	Neisseria meningitidis (98%)	Ae	O
Campylobacteraceae	*Campylobacter concisus*	Ae	O
	Campylobacter gracilis	Ae	O
Altermonadaceae	*Alishewanella fetalis*	Ae	
Pastuerellaceae	*Haemophilus haemolyticus* (98%)	Ae	O
	Haemophilus influenzae	Ae	O
	Haemophilus parahaemolyticus	Ae	
	Haemophilus parainfluenzae (97%)	Ae	
	Terrahaemophilus aromaticivorans	An	
Moraxellaceae	*Acinetobacter lwoffii*	Ae	
Pseudomonadaceae	*Pseudomonas aeruginosa*	Ae	
Xanthomonadaceae	*Stenotrophomonas maltophilia*	Ae	
	Total Species	82	82
	Total aerobes (%)	46 (56%)	
	Total Anaerobes (%)	36 (44%)	
	Number associated with oral microbiota (%)		59(72%)

Table 9-1 Microbiological classification of species identified by clone sequence analysis. Percentage values after species names refer percentage similarity of sequence to closest related species. Metabolic classification An = Anaerobic; Ae = Aerobic. Note: only strict anaerobes are classified as such; all other species, including micro-aerophiles, facultative anaerobes and aerobes are classified as aerobes.

Glossary

Asymptotic: Being in a state of having reached a plateau

Community: A conglomeration of species or individuals which can be said to interact with each other.

Core species: Species within a diverse community which can be said to be resident within a given environment. Opp Satelite species.

Diversity: Range of different species within an environment

Eutrophic: Relating to habitat with high levels of key nutrients

In silico : Performed on or with the help of a computer.

Kingdom: Highest taxonomic group into which organisms are grouped; one of six biological categories: Archeae, Bacteria, Plantae, Fungi, Animalia

Oligotrophic: relating to habitat with low levels of key nutrients

Phylogenetic: Evolutionary relatedness of species

Planktonic: Relating to mode of living that involves individual motion. Opp. sessile

Rizosphere: That part of the environment in close proximity and relating to plant roots

Satellite species: Species within a diverse community which are not considered to resident. Such species can be said to be transient to a given environment (Opp. Core species).

Species evenness: Measure of the abundance of species relative to other species within a given community.

Species richness: Number of species detected in a given sample by a given technique

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