



**Non-invasive markers of
inflammation in Cystic Fibrosis
lung disease**

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Declaration

The author performed all of the experiments and procedures presented in this thesis unless otherwise indicated in the text.

Signed

Contents

LIST OF FIGURES	6
LIST OF TABLES	8
ABBREVIATIONS	9
ACKNOWLEDGEMENTS	12
ABSTRACT	13
STATEMENT OF HYPOTHESIS	16
1.1 CYSTIC FIBROSIS LUNG DISEASE – AN OUTLINE	18
1.1.1 CF PATHOLOGY – CLINICAL OVERVIEW	18
1.1.2 <i>CYSTIC FIBROSIS LUNG DISEASE</i>	18
1.1.3 <i>PROGRESSION OF CF LUNG DISEASE</i>	21
1.2 CFTR DEFECT	25
1.2.1 <i>THE CF GENE AND CFTR PROTEIN</i>	25
1.2.2 <i>CFTR PROTEIN</i>	27
1.2.4 CFTR FUNCTION	31
1.3 <i>CF LUNG DISEASE AND AIRWAY SURFACE LIQUID</i>	35
1.3.1 <i>EVOLUTION OF LUNG DISEASE</i>	35
1.3.2.1 <i>THE HYDRATION HYPOTHESIS (LOW ASL VOLUME HYPOTHESIS)</i>	37
1.3.2.2 <i>THE HIGH SALT HYPOTHESIS</i>	38
1.3.2.3 <i>DIFFICULTIES IN ASSESSING ASL ION COMPOSITION</i>	38
1.4 <i>GENE THERAPY FOR CYSTIC FIBROSIS</i>	40
TABLE 2. ADVANTAGES AND DISADVANTAGE OF CF GENE THERAPY	41
1.4.3 PROOF OF PRINCIPLE FOR TRANSFECTION	42
1.4.4 <i>TARGET CELL POPULATION</i>	42
1.4.5 <i>GENE THERAPY FOR CF</i>	45
1.4.5.1.3 VIRAL GENE THERAPY VECTORS	47
1.4.5.2 <i>Target Transfection efficiency</i>	48
1.4.6 UNITED KINGDOM CYSTIC FIBROSIS GENE THERAPY CONSORTIUM.	48
1.5 <i>NON-INVASIVE SAMPLING TECHNIQUES IN LUNG DISEASE</i>	52
1.6 <i>MARKERS OF INFLAMMATION IN CF LUNG DISEASE</i>	58
1.6.1 <i>INFLAMMATORY RESPONSE</i>	58
1.6.1.1 <i>NEUTROPHIL RESPONSE</i>	58
1.6.1.2 <i>APOPTOSIS AND NECROSIS IN THE CF AIRWAY</i>	59
1.6.2 <i>POTENTIAL BIOMARKERS OF INFLAMMATION TO BE INVESTIGATED</i>	59
1.6.3 <i>PROTEOMICS AND NOVEL PROTEIN DISCOVERY</i>	62

<i>1.7 INTRODUCTION - SUMMARY</i>	64
2.0 SAMPLE COLLECTION AND PREPARATION	65
2.1 THE IDEAL MARKER	66
2.1.1 HYPOTHESIS	66
2.1.2 THE OPTIMAL BIOLOGICAL SAMPLE	66
2.1.4 THE IDEAL BIOMARKER	67
2.1.5 SUBJECTS	68
2.2 SAMPLE COLLECTION TECHNIQUES	69
2.2.1 EXHALED BREATH GAS ANALYSIS	69
2.2.2 Exhaled Breath Condensate collection	69
2.2.3 EBC COLLECTION DEVICES	70
2.2.3.4.1 DISINFECTING TEFLON PERFLUOROALKOXY (PFA) TUBE	71
2.3 INDUCED SPUTUM COLLECTION	76
2.3.1 INDUCED SPUTUM COLLECTION TECHNIQUE	76
2.3.2 INDUCED SPUTUM PROCESSING	76
2.5 FREEZE DRYING	80
2.7 BIOINFORMATICS TOOLS USED	84
2.8 ASSAY TECHNIQUES – CHAPTERS 3 - 6	85
3.0 – CHEMICAL ASSAY TECHNIQUES	87
3.1 CHEMICAL ASSAYS	87
3.1.0 EXHALED CARBON MONOXIDE	87
3.2 NITRIC OXIDE	90
3.3 NITRITE	93
3.4 TOTAL PROTEIN	96
4.0 – EBC - ENZYME IMMUNOASSAY TECHNIQUES	100
4.0.1 ENZYME LINKED IMMUNOSORBANT ASSAY TECHNIQUES	101
4.1 PROTEINS	101
4.1.2.1.1 STANDARD CURVE DILUENTS	102
4.2 EBC IL 8 IN CF VS CONTROL ADULTS.	115
4.5.1 LIPID MEDIATORS	120
5.0 EBC - ION MEASUREMENTS	129
5.1 pH	130
5.1.3 RESULTS	134
5.2 AMMONIUM	140
5.2.2 METHODS	141
5.2.2.1 ION SELECTIVE ELECTRODES	141

5.2.3 RESULTS	144
5.3 DISCUSSION – pH AND AMMONIUM DATA COMBINED.	149
5.4 SODIUM, CHLORIDE, POTASSIUM (AND LACTATE) IONS	153
5.4.1 METHODS	153
5.4.2 RESULTS	155
5.5 ANALYSIS OF COMBINATION OF EBC BIOMARKERS	161
6.0 – PROTEOMICS TECHNIQUES	167
6.1.1.2 OPTIMISED CHIP/BINDING BUFFER CONDITIONS	172
6.1.3 SELDI PROTOCOLS	173
6.1.4 SELDI DATA ACQUISITION	174
6.1.5 SELDI DATA ANALYSIS	175
6.2 EXHALED BREATH CONDENSATE – SELDI ANALYSIS	177
6.5 INDUCED SPUTUM – SELDI ANALYSIS	192
6.6 PROTEIN IDENTIFICATION	198
6.6.1 POLYACRYLAMIDE GEL ELECTROPHORESIS	198
6.6.4 MASS SPECTROMETRY TECHNIQUES FOR PROTEIN IDENTIFICATION	200
6.6.5 PROTEIN IDENTIFICATION USING SELDI	200
7.0 FINAL DISCUSSION CHAPTER	209
7.8 CONCLUDING REMARKS AND FUTURE DIRECTIONS	214

List of Figures

Figure 1. Life expectancy of cystic fibrosis patients	19
Figure 2. Rising % of patients colonised by pseudomonas with age	22
Figure 3. Chest Xray of normal and CF subjects	23
Figure 4. CFTR gene location in chromosome 7	26
Figure 5. Artistic impression of CFTR protein	28
Figure 6. Xray crystallographic representation of CFTR dimers	28
Figure 7. Diagram representing sites of CFTR mutations	30
Figure 8. Obstruction of Bronchiole in CF lung disease	36
Figure 9. CFTR expression in the distal conducting airways	44
Figure 10. PFA tubing apparatus	72
Figure 11. Patient using PFA apparatus	72
Figure 12. Diagrammatic representation of Jaeger cooling system	74
Figure 13. Jaeger Mouthpiece and EBC collection chamber	74
Figure 14. Subject breathing through jaeger collection device	74
Figure 15. Box plot representation of data.	83
Figure 16. Exhaled CO levels in controls and CF subjects	89
Figure 17. Exhaled NO levels in controls and CF subjects	92
Figure 18. EBC Nitrite levels in controls and CF stable subjects	95
Figure 19. EBC total protein levels in controls and CF stable subjects	98
Figure 20. Interleukin 8 standard curve – EBC and biosource buffers	104
Figure 21. Interleukin 6 standard curve – EBC and biosource buffers	105
Figure 22. Interleukin 8 standard curve with different diluents	108
Figure 23. 20pg spike of interleukin 8 in EBC	113
Figure 24. Interleukin 8 levels in EBC of control and CF subjects	116

Figure 25. EBC 8-Isoprostane levels in control and CF subjects	124
Figure 26. EBC nitrotyrosine levels in control and CF subjects	127
Figure 27. pH Boy ion selective electrode	132
Figure 28. EBC pH levels in adult control and CF subjects	137
Figure 29. EBC pH levels in child control and CF subjects	138
Figure 30. Jenway ion selective electrode	143
Figure 31. Standard curve generation for ammonium chloride	143
Figure 32. EBC ammonium levels in adult control and CF subjects	147
Figure 33. EBC ammonium levels in child control and CF subjects	148
Figure 34. EBC ion levels in control and CF subjects	157
Figure 35. EBC ratio between ions in control and CF subjects	158
Figure 36. Lung function parameter – FEV1 in control and CF subjects	162
Figure 37. Biomarker decision tree for combination of markers	164
Figure 38. Application of sample to SELDI chip surface	170
Figure 39. Chip surfaces used in this thesis	170
Figure 40. Diagram representing time of flight and signal intensity	171
Figure 41. SELDI software generated data views	171
Figure 42. Contamination evident in EBC	180
Figure 43. Similar profiles of EBC and BAL	180
Figure 44. Mass shift effect of EBC on recombinant interleukin 8	182
Figure 45. 6 example proteins detected in BAL using SELDI	188
Figure 46. Top 6 examples of induced sputum biomarkers using SELDI	196
Figure 47. Peptide profile of protein 10589 digest (calgranulin A)	203

List of Tables

Table 1. Non respiratory pathological changes of CF	24
Table 1. Advantages and disadvantages of CF gene therapy	41
Table 2. Comparison of three techniques to sample ASL	53
Table 3. Comparison of three techniques to sample ASL	53
Table 4. EBC pH adult data	136
Table 5. EBC pH childrens data	136
Table 6. EBC pH combined adults and childrens data	136
Table 7. EBC ammonium adult data	146
Table 8 . EBC ammonium childrens data	146
Table 9. EBC ammonium combined adults and childrens data	146
Table 10. EBC sodium levels in control and CF subjects	156
Table 11. EBC chloride levels in control and CF subjects	156
Table 12 EBC potassium levels in control and CF subjects	156
Table 13. EBC lactate levels in control and CF subjects	156
Table 14. Post-translational modifications at mass range 238-272	183
Table 15. SELDI BAL data – lead 21 proteins	187
Table 16. SELDI Induced sputum data – lead 38 proteins	195

Abbreviations

aaV	Adeno-associated virus
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
ASL	Airway surface liquid
BAL	Bronchoalveolar lavage
BHT	Butylated Hydroxy Toluene
BSA	Bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
CBAVD	congenital bilateral absence of the vas deferens
CF	Cystic fibrosis
CFBEs	Human Cystic Fibrosis Bronchial Epithelial Cell line
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl ⁻	Chloride Ion
CO ₂	Carbon Dioxide
COPD	Chronic Obstructive Pulmonary Disease
Da	Dalton
DNase	Deoxyribonuclease (Recombinant)
DTT	dithiothreitol
EBC	Exhaled Breath Condensate
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ENaC	Epithelial sodium channel (amiloride sensitive)
ER	Endoplasmic Reticulum
FEV ₁	Forced Expiratory Volume in 1 second
GL67	Genzyme lipid 67

GM 1	Asialoganglioside membrane receptor 1
GSH	Glutathione (oxidised)
GSSG	Glutathione (reduced)
GST	Glutathione S Transferase
GTAs	Gene transfer agents
H ⁺	Hydrogen Ion concentration
HBD	Human Beta Defensin
hBEs	Human Bronchial Epithelial Cell line
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IL	Interleukin
INF α	Interferon alpha
IFN γ	Interferon gamma
iNOS	inducible Nitric Oxide Synthetase
IQR	Inter-quartile range
IS	Induced Sputum
K ⁺	Potassium ion
kDa	kilo Dalton
l	litre
LPS	lipopolysaccharide
MALDI TOF	Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
NED	N-1-Naphthyl-ethylenediamide dihydrochloride
PBS	Phosphate buffered saline

RT-PCR	Reverse transcriptase polymerase chain reaction
SA 100/8	Calgranulin A
SDS	Sodium dodecyl sulphate
SELDI TOF	Surface Enhance Laser Desorption/Ionisation Time of Flight Mass Spectrometry
SeV	Sendai virus
TNF	Tumour Necrosis Factor
Triton	t-Octylphenoxypolyethanol

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Abstract

Introduction

Cystic fibrosis (CF) lung disease is characterised by early airways infection and inflammation, chronic suppuration, frequent infective exacerbations and an increased influx of acute, and chronic inflammatory cells. The inflammatory process involves activation of many cell types including neutrophils, macrophages and epithelial cells, and leads ultimately to the development of progressive respiratory failure and death. Accurate assessment of the inflammatory process is a crucial part of disease monitoring and should allow appropriate evaluation of therapeutic interventions so as to maximize control of the respiratory sequelae of the disorder.

Lung function markers such as FEV₁ are insensitive and indirect. Direct but invasive methods such as fiberoptic bronchoscopy and biopsy are limited in application, repeatability and safety. Non-invasive methods of assessment are, therefore, attractive. Exhaled Breath Gases, Exhaled Breath Condensate and Induced Sputum provide potential for such measures. These techniques are safe, simple, repeatable and could assess all airways and can be used in children as young as 6 years. We hypothesised that biomarkers of inflammation in Cystic Fibrosis Lung Disease are measurable in samples collected non-invasively, and can be developed into clinically useful assays. These assays would have the ability to reflect the level of inflammation in the CF lungs as well as holding the potential to act as surrogate markers of CFTR function.

Methods

Exhaled breath gases, exhaled breath condensate, bronchoalveolar lavage fluid and induced sputum were investigated using a number of analysis techniques to identify the markers which best discriminated CF from non CF subjects. Analysis techniques used were electrochemical cells, chemiluminescence, ELISA, EIA, ion selective probes and mass spectrometry.

Results

Markers found to discriminate CF from non CF subjects were EBC pH and ammonium, and 38 proteomic markers were found in induced sputum. 21 proteomic markers were found in bronchoalveolar lavage fluid. One biomarker has been identified with confidence, Calgranulin A.

Discussion

A large component of the work of this thesis was focussed on exhaled breath condensate. Two markers, pH and Ammonium were different between the CF and control groups. The measurement of EBC pH and ammonium as markers of inflammation should be used in future gene therapy trials as they are cheap, quick and simple to perform

Using clean techniques free from contamination, no proteins are repeatedly detectable in EBC using highly sensitive SELDI techniques. This technique reflects the highest sensitivity of any available proteomics instrument and therefore until new technologies become available, it would be incorrect to assay any proteins in EBC.

The induced sputum proteomics study identified 38 independent markers of CF lung inflammation. Therefore, sampling by collection of induced sputum should

be used in gene therapy trials. The endpoints should be assessed by a combination of SELDI as an endpoint and by ELISA where this is available.

The marker Calgranulin is likely to report on neutrophil recruitment to the lung. It is anticipated that this will be a sensitive marker of inflammation in the lung and it also has the potential to report on successful of gene transfer as it is raised in heterozygote carriers as well as homozygotes with CF.

Therefore, the non-invasive technique induced sputum coupled to proteomic analysis would have the ability to reflect the level of inflammation in CF subjects and may also report on CFTR function.

Statement of Hypothesis

Markers of inflammation in Cystic Fibrosis Lung Disease are measurable in samples collected non-invasively, and can be developed into clinically useful assays.

These assays would have the ability to reflect the level of

1. Inflammation in the CF lungs.
2. CFTR function – this would be sensitive enough to detect improvement following administration of gene transfer agents.

1.0 - Introduction

1.1 Cystic Fibrosis Lung Disease – An Outline

Cystic Fibrosis (CF) is the most common, fatal single gene defect in the Caucasian population. It is an autosomal recessive condition caused by a mutation of the CF gene on chromosome 7. The CF Gene encodes for the cell membrane ion transport protein Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Individuals of Northern European origin have the highest incidence of CF with about 1 in 2500 newborn children affected and a carrier frequency of 1 in 25. There are approximately 70,000 affected individuals in the world, with 7500 living in the UK. The commonest mutation is the $\Delta F508$ mutation – a 3 base pair DNA deletion that results in the deletion of phenylalanine at position 508 in the protein. Cystic Fibrosis affects the respiratory, alimentary and reproductive tracts. CF heterozygotes are considered phenotypically normal. The disease is much less common in African and Asian populations, where carrier frequencies of 1/100 to 1/200 have been estimated. Precise incidence in the developing world is not known due to high infant mortality rates and limited survival of CF homozygotes (Greening, 2000).

Median life expectancy for affected individuals has improved from 2 years in 1940, to 30 years in 2000, as shown compared to average life expectancy (Figure 1). It has been predicted that with current rates of improvements in therapies, affected children born in the year 2000 have an average life expectancy around 40 years.

1.1.1 CF Pathology – Clinical overview

Cystic Fibrosis is a multisystem disorder. The major cause of morbidity and mortality is CF Lung Disease. Subjects can also suffer significant pathology of the alimentary and reproductive tracts.

1.1.2 Cystic Fibrosis Lung Disease

Since the cloning of the CF gene, it has become accepted that many of the pathophysiological abnormalities are related to abnormal ion transport (Riordan et al., 1989, Quinton, 1999).

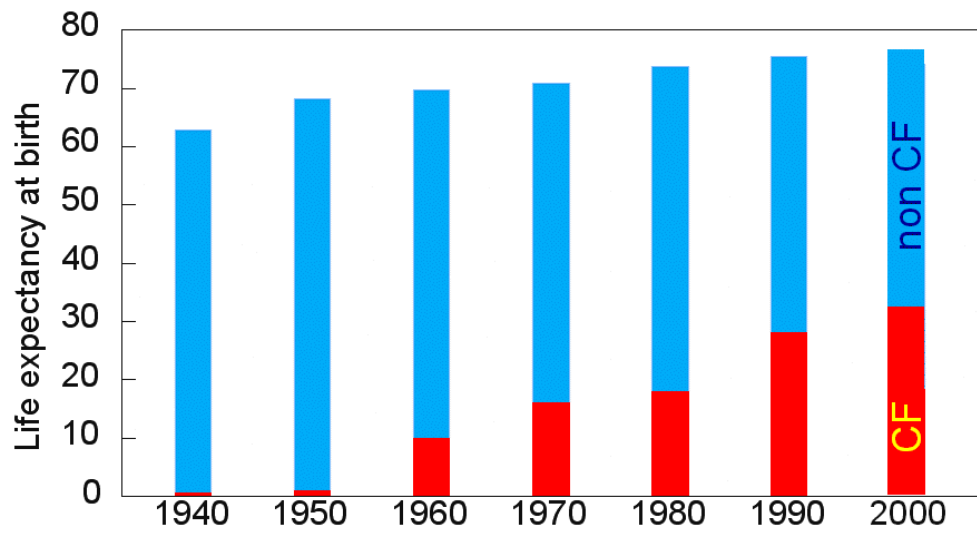


Figure 1. Life expectancy of Cystic Fibrosis patients.

Red bar denotes CF subjects and Blue bar non-CF subjects. Data derived from UK census data. (Dodge et al., 2007)

Improvement in life expectancy for subjects with cystic fibrosis (red) as compared to the increase in life expectancy in non-CF subjects from 1940 to 2000 in the UK.

In Cystic Fibrosis neonates, the lungs appear pathologically normal (Sobonya and Taussig, 1986). Early infection with pathogenic bacteria is evident. The first step in this process is not entirely clear. It may be that abnormal mucociliary clearance along with reduced Airway Surface Liquid height and mucus hypersecretion is enough to permit the colonisation and impaired clearance of pathogenic bacteria. Others have argued that the initial defect is inflammation prior to infection, evidencing this with studies of bronchoalveolar lavage fluid from the lungs of neonates where proinflammatory cytokines and inflammatory cells were detected in the absence of any detectable pathogenic bacteria (Balough et al., 1995).

Consequent to the presence of pathogenic bacteria and impaired bacterial clearing, patients develop persistent suppurative lung disease. Small airways obstruct with viscous mucus, pathogenic organisms and inflammatory cells. This results in air trapping and then widespread fibrosis and bronchiectasis. Long-term suppuration and bronchiectasis lead to a decline in lung function. The lung function marker, forced expiratory volume in 1 second (FEV₁) is a predictor of prognosis. The organisms *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Stenotrophomonas Maltophilia* and *Burkholderia Cepacia* are pathogenic bacteria that colonise the airways of patients with CF.

1.1.3 Progression of CF lung disease

Initial pathology is of blockage of bronchioles with mucus, bacteria and neutrophils. Progressive fibrosis and bronchiectasis cause a decline in lung function. As the disease worsens, there are changes of colonising organisms. With milder lung disease such as *Haemophilus Influenzae* and *Staphylococcus Aureus* are found. Later organisms which cause more severe morbidity such as *Pseudomonas aeruginosa*, *Stenotrophomonas Maltophilia* and *Burkholderia Cepacia* may appear. (Figure 2) *Pseudomonas aeruginosa* is one of the most significant pathogens in CF, causing acceleration in decline of lung function and increased subsequent morbidity and mortality (Jacques et al., 1998). The changes of fibrosis and bronchiectasis can be seen radiologically (Figure 3).

1.1.4 Cystic Fibrosis pathology – non-respiratory disease

The major cause of mortality in Cystic Fibrosis is lung disease. Cystic Fibrosis pathology in other organs does contribute to significant morbidity (Table 1).

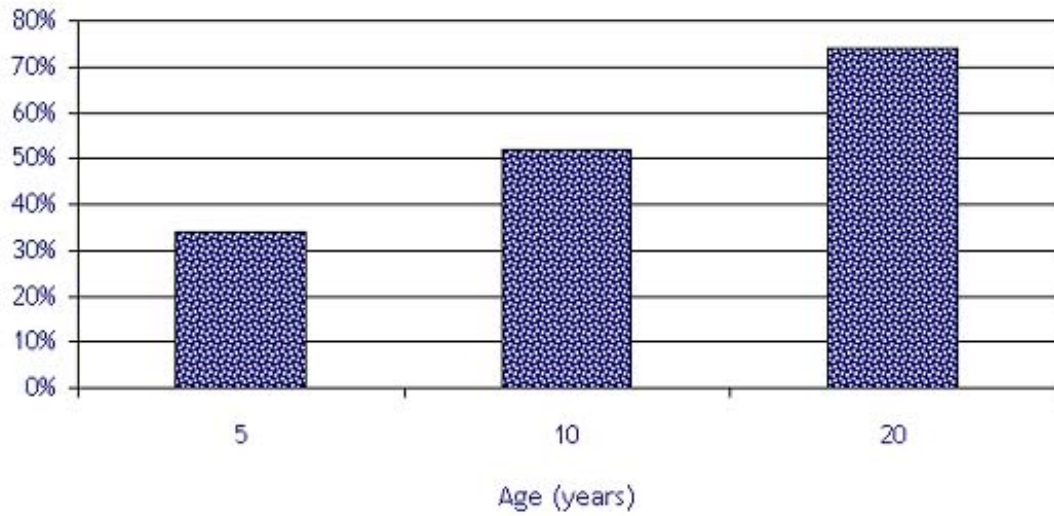


Figure 2. The rising percentage of CF patients who are colonised with *Pseudomonas aeruginosa* at age 5, 10 and 15 years of age (Luiz, 2001)

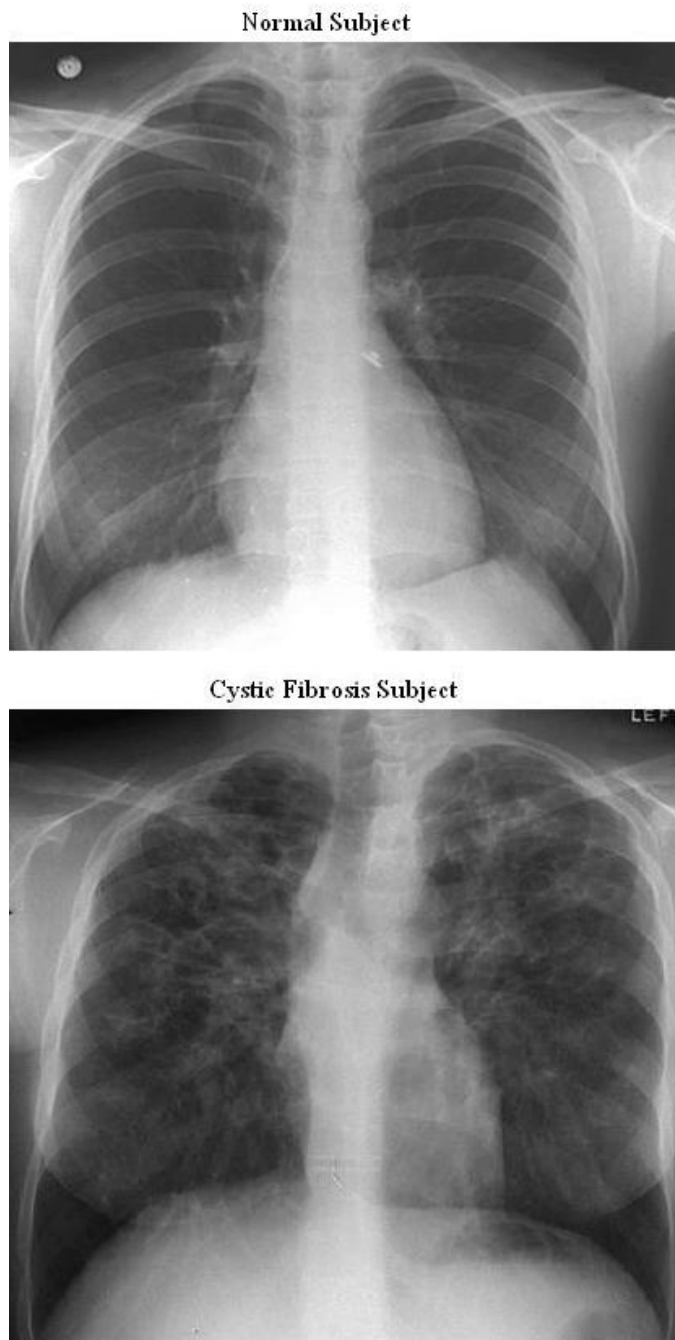


Figure 3. Chest X rays of normal and CF subjects

The lower chest x ray panel demonstrates the upper lobe fibrosis and bronchiectatic changes typical of CF lung disease compared to healthy control subject (upper X ray panel).

Organ	Pathophysiological Changes	Pathology
Sweat Duct	High sodium and chloride secretion	Salt depletion in heat
Pancreas	Decreased volume and increased concentration of secretions	Early—duct plugging, dilatation, acinar atrophy Late—fibrous and fatty replacement, and loss of islets
Intestine	Concentrated secretions, mucus altered— hyperglycosylated and hypersulfated	Meconium plug, distal ileum; crypt dilatation; meconium peritonitis; distal intestinal obstruction syndrome; constipation
Liver	Reduced bile salt secretion, increased circulating bile salt concentration	Early—bile duct hyperplasia, plugging of intrahepatic bile ducts, focal biliary cirrhosis Late—multilobular cirrhosis
Gallbladder	Reduced bile salt pool, lithogenic bile	Cystic duct occlusion, hypoplastic gallbladder, gallstones
Epididymis and vas deferens	Embryological/ developmental failure	Absent—fibrous replacement Male infertility

Table 1. Non respiratory pathological changes of Cystic Fibrosis

1.2 CFTR Defect

1.2.1 The CF Gene and CFTR Protein

CF Gene

The CF gene was identified in 1989 (Riordan et al., 1989). Earlier linkage analysis identified the putative CF locus to be on the long arm of chromosome 7 (Tsui et al., 1985), and to the band 7 cen-q22 (Wainwright et al., 1985), as shown in the following schematic (figure 4). Cloning and characterisation studies revealed the CF gene to be approximately 6500 base pairs long (the putative protein having a membrane bound component and an ATP binding domain) as well as revealing the commonest CF mutation, the deletion of phenylalanine at amino acid 508. The gene encodes the Cystic Fibrosis Transmembrane Regulator protein, which has 1480 amino acids (Riordan et al., 1989).

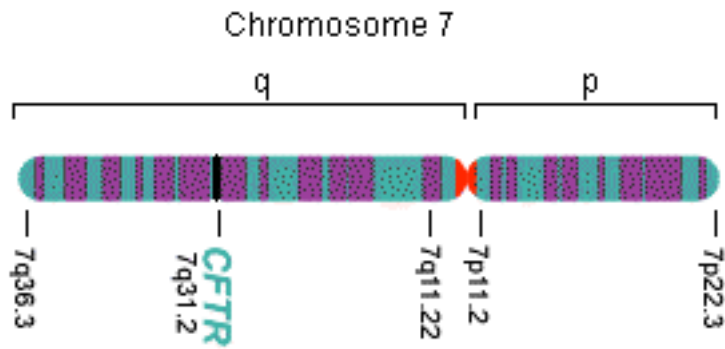


Figure 4. The location of CFTR gene in the long arm of chromosome 7 (cen-q22) is shown.

The black band represents CFTR, on the long arm of chromosome 7.

1.2.2 CFTR Protein

CFTR protein shows sequence homology with ATP-Binding Cassette (ABC) transporters (Riordan et al., 1989). Such ATP dependant ABC transporters have been identified as being cross membrane transporters of molecules (Hyde et al., 1990). CFTR is composed of five domains: two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory (R) domain (Sheppard and Welsh, 1999). Artistic impressions of the protein have been drawn (figure 5), and attempts to assess CFTR structure using recombinant protein and X ray crystallography have predicted that it forms a heterodimer in the membrane to form a pore (figure 6).

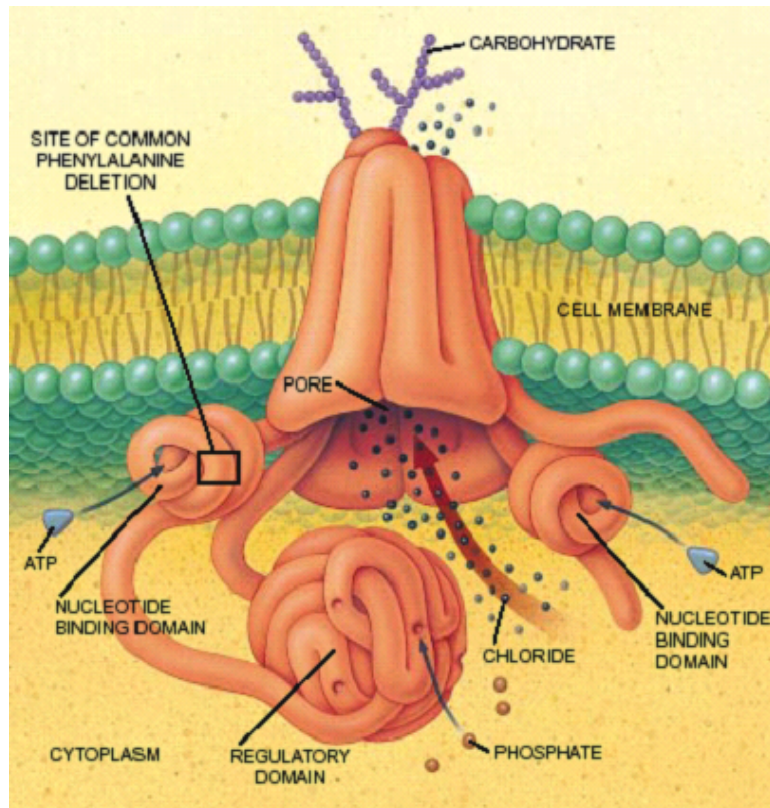


Figure 5. Artistic representation of CFTR protein

This artist's impression shows the regulator R domain lying below the ion pore (Welsh and Smith, 1995).

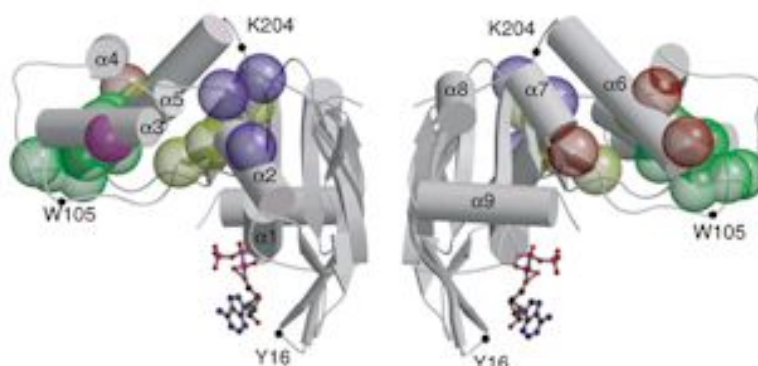


Figure 6. X ray crystallographic representation of CFTR dimers

The figure shows two CFTR protein molecules together spanning the cell membrane to form an ion pore (Hung, 1998).

1.2.3 CFTR gene mutation/protein defect

To date there are 1542 different genetic mutations recorded as being associated with the disease (Cystic Fibrosis Mutations Database - <http://www.genet.sickkids.on.ca/cftr/>).

The most common CF mutation is delta F508, which accounts for approximately 70% of worldwide CF mutations. A group of 12 further mutations are common and collectively amount for another 15% of the total. The remaining mutations are all individually rare, many having been observed in only one or a few closely related families. The mutations can be put into different classes and defects may occur at different sites within the cell (Figure 7). There is some correlation between phenotype (i.e. clinical symptoms) and genotype.

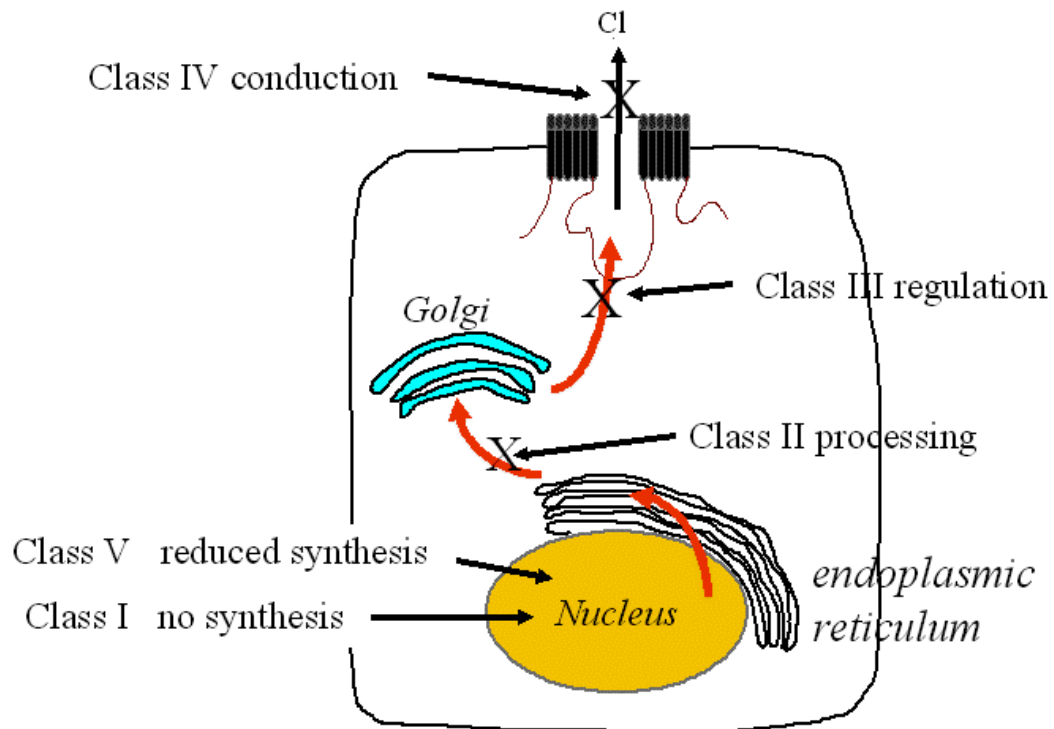


Figure 7. Diagram representing sites of CFTR mutation Class I-V.

Cartoon representation of the different sites within the cell where mutant CFTR protein defect occurs (Greening, 2000, Riordan et al., 1989).

- Class 1: Defective protein production.
- Class 2: Defective trafficking.
- Class 3: Defective regulation of CFTR.
- Class 4: Altered/defective conduction of Cl⁻ channel.
- Class 5: Reduced synthesis of functional CFTR.
- Class 6: Altered stability of mature protein.

1.2.4 CFTR Function

The main function of CFTR protein is established as being an ATP dependant chloride channel situated in the apical membrane of epithelial cells (Riordan et al., 1989).

Chloride channel activity is not the only possible function of CFTR. Other potential CFTR functions are that it acts as a bicarbonate channel (Choi et al., 2001b), that it interacts with the sodium channel ENaC (Stutts et al., 1997), and that it interacts with inflammatory mediators (Balough et al., 1995) and bacteria (Zar et al., 1995). Despite considerable effort, there is no unifying hypothesis, which explains the link between CFTR protein dysfunction and the pathological changes of Cystic Fibrosis lung disease.

1.2.4.1 CFTR as a chloride channel.

Evidence that Cystic Fibrosis was a disease of an ATP dependant chloride channel was first shown using sweat duct perfusion studies – abnormally low Cl⁻ permeability in cystic fibrosis sweat ducts led to poor Na⁺Cl⁻ reabsorption. Such electrophysiological evidence predates the cloning of the gene (Quinton, 1983, Schoumacher et al., 1987). This was later confirmed along with the identification of the requirement for cAMP dependant protein kinase activity (Hwang et al., 1989). After discovery of the CF gene, within a year, proof of principle for retroviral gene therapy correction of the chloride defect was obtained *in vitro* (Drumm et al., 1990).

Confirmatory evidence that CFTR acts as a chloride channel are studies showing reduced chloride conductance in the human CF nose which can be detected by nasal potential difference (PD) (Knowles et al., 1995).

1.2.4.2 CFTR as a bicarbonate channel.

Acidification of secretions in Cystic Fibrosis was first identified in 1987. Researchers investigating sweat pH found it to be reduced in CF subjects, but this was thought to reflect rate of sweat production, and passive reabsorption of bicarbonate (Bijman and Quinton, 1987). CFTR functioning as both a chloride and a bicarbonate pore in the pancreas was then detected (Durie, 1992). Later, confirmation of diminished bicarbonate secretion in porcine airway was found, and this reduction in bicarbonate secretion was attributed to submucosal gland CFTR dysfunction (Ballard et al., 1999). When a correlation between pancreatic function and CFTR bicarbonate secretion was

found, the authors proposed that diminished bicarbonate secretion might in fact be the defect causing CF pathology. They argued that acidic secretions of CF subjects would cause increases in mucus viscosity and bacterial binding, leading to CF lung disease (Choi et al., 2001a, Choi et al., 2001b, Reddy and Quinton, 2001). Reasoning that defective bicarbonate secretion could be the basic defect came with the discovery that CF airway cells failed to react to an acid load being applied to the ASL, whereas wild type airway cells rendered the ASL alkaline in response (Coakley et al., 2003).

1.2.4.3 CFTR interaction with the Sodium channel ENaC.

Potential difference measurements revealed not only chloride channel activity, but evidence that the basal rate of sodium absorption was raised in CF polyps, when compared with control tissues. Increased sodium absorption was found to be due to the epithelial sodium channel (ENaC), confirmed by blocking the activity with amiloride (Boucher et al., 1986). The presence of CFTR in the membrane made it more likely that the ENaC pore would not be open. Therefore, it has been established that in CF cells, through loss of the direct interaction of CFTR with ENaC at the level of single channel gating, the sodium channel pore remains open and sodium hyperabsorption occurs (Stutts et al., 1997, Zar et al., 1995).

1.2.4.4 CF and increased bacterial binding

Patients with Cystic Fibrosis have a predilection for infection with the organisms *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. The interaction between CFTR and *Pseudomonas aeruginosa* has been most studied and best defined. Adherence of *Pseudomonas aeruginosa* to epithelial cells from patients with CF was found to be greater than adherence to the cells of control subjects and heterozygotes (Zar et al., 1995). This increased adherence was found to be related to activation of the asialoglycoprotein receptor asialo GM1, whose expression is increased in regenerating epithelial cells, which functions as a *Pseudomonas aeruginosa* pilin receptor. It was postulated by this group that Cystic Fibrosis cells would undergo increased epithelial repair, and that it is the repair event itself which in turn exposes the asialoGM1 receptor, allowing increased *pseudomonas* binding (Bryan et al., 1998, de Bentzmann et al., 1996, Balough et al., 1995, Konstan and Berger, 1997).

1.2.4.5 CF and inflammatory mediators

The argument that the initial abnormality in Cystic Fibrosis could be inflammation prior to bacterial infection was evidenced by Bronchoalveolar Lavage (BAL) studies of neonates. These studies revealed that inflammatory cells and proinflammatory cytokines could be detected before evidence of bacterial colonisation/infection (Balough et al., 1995, Konstan and Berger, 1997), suggesting that the initial inflammation may be the causative factor in CF lung disease, rather than as a response to pathogenic bacteria. It has, however, been noted that markers of CF lung damage such as FEV₁ correlate with bacterial infection, but not with proinflammatory cytokine levels (Nixon et al., 2002).

1.2.4.6 Interaction with modifier genes

It has been argued that the lung disease associated with CF reflects the interaction between CFTR protein and other genes and proteins. This interaction could be the basic defect, or alternatively the severity of CFTR dysfunction may be modified by other genes and proteins.

One particularly compelling argument is centred on the clinical heterogeneity of CF subjects, as each genotype poorly correlates with disease phenotype with regard to lung disease. It may, therefore, be that Cystic Fibrosis lung disease is not related to an ion channel defect, but rather CFTR effects on other “modifier” genes and proteins.

Further evidence for genetic “modifiers” is the discordance in lung function in dizygotic twins, and greater concordance in lung function seen in monozygotic twins, suggesting CF lung disease severity is modified by an inherited gene component in addition to the CFTR gene itself (Mekus et al., 2000). The concept of modifier genes and protein products that interact with CFTR is currently gaining momentum with the investigation of the CFTR interactome, i.e. the set of proteins that interact with CFTR and determine net function in an organ specific fashion.

The best evidence for such interactions is the discovery of proteins that interact with the intracellular components of the C terminus of CFTR. Molecules such as EBP50/NHERF1 and ezrin have been shown to interact with the C terminus. Whilst their function is poorly understood, it is known that these molecules then interact with

other intracellular proteins such as actin (Haggie et al., 2004). It is conceivable therefore, that a function unrelated to Chloride channel efflux may well lead to CF lung disease. Other examples of genes implicated with CFTR function are mannose-binding lectin, glutathione-S-transferase, transforming growth factor-beta1, tumor necrosis factor-alpha, beta2-adrenergic receptor, and HLA class II antigens (Merlo and Boyle, 2003).

1.2.4 Correction of CFTR function

As the cellular ramifications of how CFTR dysfunction leads to CF lung disease have not yet been clearly established, the best potential for development of novel therapies may lie in inserting “healthy” Wild Type CFTR into the cell membrane, by CF Gene Therapy.

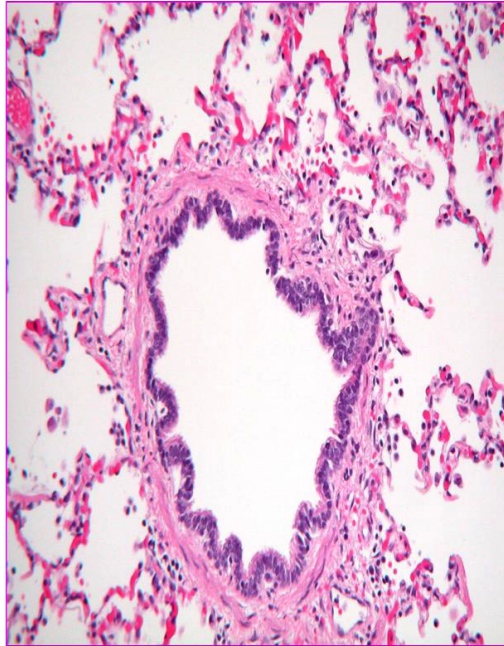
1.3 CF Lung Disease and Airway Surface Liquid

1.3.1 Evolution of lung disease

The clinical course of Cystic Fibrosis lung disease varies greatly between individuals. The genotype does not accurately predict the severity and prognosis of CF lung disease. Some individuals may present soon after birth with CF pathogens in the lung, whereas other individuals with the same genotype may present in adolescence and adult life, reflecting differing environmental exposure, path of care as well as genetic factors.

The major pathology is exhibited in the airway lumen - airway obstruction with blockage by mucus, neutrophils and bacteria (Figure 8). Analysis of the Airway Surface Liquid (ASL) in this lumen could therefore be used for discovery of biomarkers of CFTR function and of CFTR related inflammation.

Normal Lung Histology



CF Lung Histology

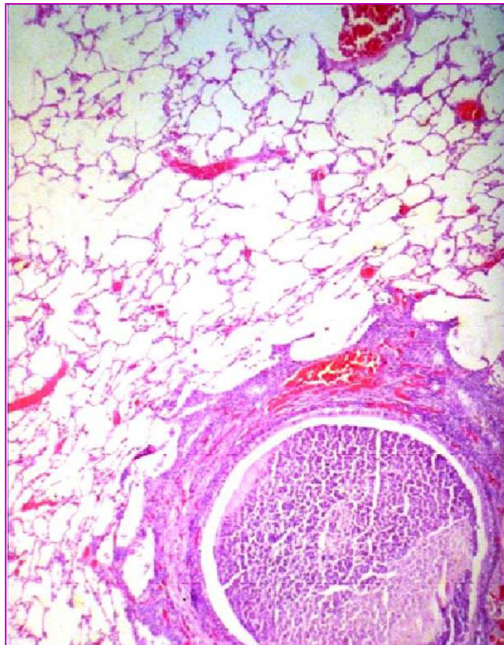


Figure 8. Obstruction of Bronchiole in CF lung disease (different magnification)

These images are of histological sections through a normal lung (upper image) and CF lung (lower image). The lumen of the largest bronchus in the CF subject can be seen to be distended and obstructed – by mucus, neutrophils and bacteria.

1.3.2 Alterations to the Airway Surface Liquid

The airway surface liquid in Cystic Fibrosis is altered for at least two reasons:

- Altered mucus composition as a direct consequence of the ion channel defect.
- In response to the infective and the inflammatory component of CF.

Alterations in mucus composition

The basic defect of reduced chloride efflux and increased sodium influx into the cell has given rise to two theories as how the chloride channel defect might precipitate lung disease.

1.3.2.1 The hydration hypothesis (low ASL volume hypothesis)

This hypothesis (Boucher, 2003, Boucher, 2004, Matsui et al., 1998, Tarran et al., 2001) proposes that the most important control for Airway Surface Liquid height is the absorption of sodium ions. This is accompanied by the diffusion of chloride ions into the cells via tight junctions. In CF, the sodium absorption is accelerated and chloride efflux defective, leading to an increased rate of water absorption. Reduced water in the ASL would lead to depletion of a specific compartment of the ASL - the periciliary liquid (PCL). This would then lead to defective ciliary clearance and reduced ciliary beat frequency, reducing the efficacy of the mucociliary escalator in clearing mucus from the lung. Subsequent to PCL depletion, mucus adheres to airway surfaces and persistent mucin secretion generates the formation of "thickened" mucus plaques and plugs. These plaques and plugs would be a nidus for persistent bacterial colonisation. In the case of *Pseudomonas aeruginosa*, these bacterial plaques allow formation of mucoid colonies in a biofilm, conferring bacterial resistance to both host defences and standard antibiotic regimens. The most compelling data supporting this hypothesis are the images from Air Liquid Interface cultures and electron microscopy showing shortening of the PCL height in cultures of CF epithelial cells from CF patients (Boucher, 2003, Boucher, 2004).

1.3.2.2 The high salt hypothesis

This hypothesis postulated that in normal ASL the fluid would be hypotonic and in CF the ASL would be isotonic. The increased salinity of CF ASL would have a detrimental effect on activity of antibacterial proteins such as defensins, lysosymes, cathelicidins and lactoferrin. This relies on the premise that the epithelium is impermeable to water and that tight junctions do not allow absorption of water, whilst transport of sodium and chloride continue actively through CFTR and ENaC. This theory suggests that CFTR regulates chloride transport across the apical membrane of epithelial cells, as confirmed by air-liquid interface cultures (Uyekubo et al., 1998). Therefore, as this regulation is deficient in Cystic Fibrosis, chloride levels would be raised in the extracellular fluid in CF. The evidence for this theory relies on the findings that CF ASL sodium and chloride levels were increased and that bacterial killing by CF ASL was impaired (Smith et al., 1996). Since then however, contradictory reports have shown that high salt does not diminish the antibacterial activity of ASL, partly invalidating the high salt ASL hypothesis (Bals et al., 2001, Matsui et al., 1998, Travis et al., 2000).

1.3.2.3 Difficulties in assessing ASL ion composition

Confirming whether the low salt or the isotonic salt hypothesis is correct depends upon *in vivo* assaying of sodium and chloride levels of ASL, but this is technically difficult. Sampling of airway surface liquid by conventional bronchoalveolar lavage would affect the levels of ions due to dilution effect and the use of isotonic sodium chloride solution for the lavage. Sampling using filter paper and bead approaches to absorb ASL have instead been used. There is no consistency in the results of the approaches used, perhaps reflecting methodological variations in collection of ASL. These variations could include mixing of periciliary liquid, mucus, secretions from submucosal glands and perhaps active secretion of ions and fluid - as insertion of filter paper etc. onto the airway may not be entirely inert. Investigators have found results, which generally support their hypothesis with regard to low salt and isotonic salt. Knowles et al found no differences in ASL ion composition (Knowles et al., 1997), whereas Quinton's group found ASL to be hypotonic, but isotonic in Cystic Fibrosis (Joris et al., 1993).

The compelling evidence of Boucher's group showing images of reduced ASL height in CF airway cells cultured on air-liquid interface casts doubt on the validity of the low salt hypothesis (Boucher, 2003).

1.4 Gene Therapy for Cystic Fibrosis

Improvements in conventional therapies have led to an increase in life expectancy, with predictions that a child born in 2007 has a median life expectancy of approximately 40 years. However, there is clearly a need for more effective therapies and since the isolation of the cystic fibrosis (CF) gene in 1989 (Kerem et al., 1989), the potential for gene therapy to correct the basic defect of this disease has been investigated.

1.4.1 Barriers to successful CF gene therapy

As the major cause of mortality is CF lung disease, the primary target for gene therapy is the respiratory epithelium of the lung. This target for gene therapy offers several advantages and disadvantages (Table 2).

1.4.2 The ideal gene transfer agent

The ideal gene transfer agent would be deliverable topically using an inhaled delivery device, exhibit a high level of transfection efficiency, transfect the putative stem cell population of the lung, have low toxicity and cause long term expression of wtCFTR at levels similar to exogenous levels. No currently available gene transfer agents or clinical protocols achieve all of these ideals.

Advantages of CF Gene Therapy	Disadvantages of CF Gene Therapy
Lung epithelium accessible to topical delivery	Respiratory epithelium evolved to resist exogenous DNA
Lung is the target organ	Stem cell population of lung inaccessible/unknown
Gene transfer in vitro corrects chloride channel defect	Detection of effective therapy is difficult
5% transfection efficiency may be satisfactory	Mucus and inflammation may block transfection in the adult population (the initial treatment population)
Mouse models demonstrating electrophysiological abnormality can be studied	No animal model demonstrates CF lung disease (pathology)
Corrects the primary CFTR defect	Most pathology represents secondary disease/damage, not the primary CFTR defect

Table 2. Advantages and disadvantage of CF Gene Therapy

1.4.3 Proof of Principle for transfection

Within a year of identification of the CF gene, proof of principle that gene therapy could correct the ion channel defect was shown in cell lines, and patch clamping showed correction of anion efflux (Drumm et al., 1990). Prolonged correction of the genetic defect was performed in proliferating cells using retrovirus (Olsen et al., 1992). Transfer of human CF gene to the mouse airway was first demonstrated in 1992 (Rosenfeld et al., 1992). Topical delivery of recombinant adenovirus was used in non human primates and transgene transfection was detected particularly in alveolar cells (Engelhardt et al., 1993). Lack of repeatable delivery of viral gene therapy was identified as a problem due to both T cell immunity and the presence of neutralising antibodies (DeMatteo et al., 1997, Yang et al., 1995). Recent gene therapy trials have used AAV2 and have shown reduction in induced sputum interleukin 8 at 30 days (Moss et al., 2004). Despite modulation of the vector and the immune response, no viral gene therapy agent has been shown to have the ability to deliver cDNA to the airway epithelium repeatedly.

Evidence for functional gene transfer in the CF mutant mouse using liposomes rather than viruses was first demonstrated in 1993 (Alton et al., 1993, Hyde et al., 1993). Subsequently, phase I clinical trials were performed in the human nose (Caplen et al., 1995, Gill et al., 1997, Porteous et al., 1997) and in the human lung (Alton et al., 1999). Delivery of liposomal gene therapy to the nose and lung of adults produced evidence of correction of the chloride defect, as well as a reduction in bacterial adherence (Alton et al., 1999). These studies provided evidence of correction of the ion transport defect in some patients, however this correction was short lived. Subsequent improvements in efficacy and repeatability would be required for clinical benefit.

1.4.4 Target Cell Population

Potential targets for gene therapy in the airway include ciliated, non-ciliated and goblet cells in the surface airway epithelium as well as submucosal glands. As yet, the stem cell population of the lung has not been identified, but if/when, it is identified, it would be the ideal target cell population for transfection, as potentially lifelong gene

transfer/expression could occur. At present, however, it is unclear which cellular targets are most relevant with regard to gene therapy.

1.4.4.1 Ciliated epithelial cells of the conducting airways

Cystic Fibrosis presents as a small airway disease. It is therefore plausible that airway epithelial cells in the conducting airways are the most relevant target for CF Gene Therapy (Jiang and Engelhardt, 1998). CFTR expression in the distal conducting airways is higher than in the proximal bronchi. It is apically localised in epithelial cells (Engelhardt et al., 1994). Access to epithelial cells of the conducting airways is possible topically through aerosol-inhaled delivery.

The Engelhardt study used in situ hybridization and immunohistochemistry to quantify CFTR expression in the distal conducting airways – high levels of expression were found in the airway cells spanning the terminal bronchioles and respiratory bronchioles (Engelhardt et al., 1994). These cells are likely to be most abundant at the 15-25th generation of division of the airway (Figure 9).

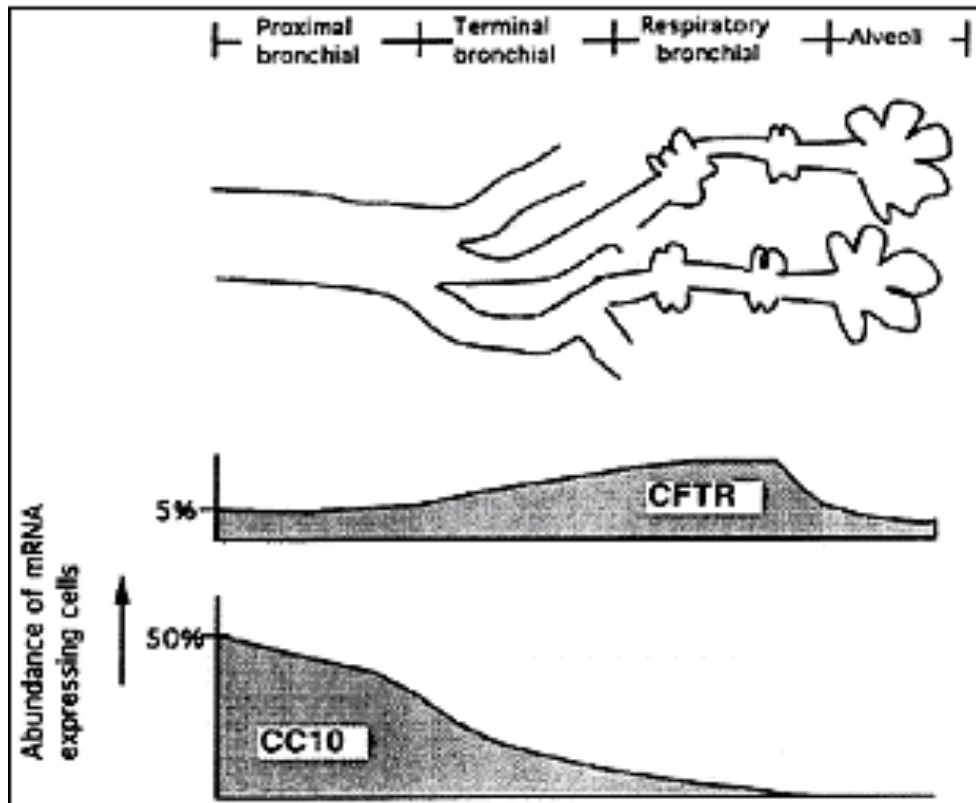


Figure 9. CFTR expression in the distal conducting airways (Engelhardt et al., 1994)

This figure is taken from the publication by Engelhard (Engelhardt et al., 1994) showing the expression of CFTR was greatest in the terminal and respiratory bronchiole i.e. the distal bronchial tree with a marked fall in expression of CFTR mRNA in the alveoli. The implication of this figure is that the most appropriate target for CFTR gene transfer would be in the terminal and respiratory bronchiole. Clara cell 10kDa protein (CC10) expression was compared as a control as it was most abundant in the proximal bronchioles.

1.4.4.2 Submucosal Glands

Work has shown high levels of CFTR expression in the submucosal glands and that their secretions are the principal contributor to the ASL of the porcine trachea (Ballard et al., 1999). They suggested that submucosal gland CF dysfunction leading to dehydrated viscous mucus is the main contributor to CF lung disease. However, as submucosal glands are only present to the level of cartilaginous rings (i.e. first few generations of airway – to approximately 3.5mm in diameter), and CF lung disease starts in the small conducting airways which are more distal (Sobonya and Taussig, 1986) submucosal glands cannot contribute to the site of initial pathology.

The ciliated and non-ciliated cells of the distal conducting airways have been chosen as the primary target of CF gene therapy by the UK Cystic Fibrosis Gene Therapy Consortium.

1.4.4.3 Stem Cell population of the lung

Other successful gene therapies (e.g. in human Severe Combined Immunodeficiency (SCID)) have targeted bone marrow stem cells using viruses and have brought about long term correction of the genetic defect (Hacein-Bey-Abina et al., 2002).

The stem cell population of the lung has not yet been identified (Cardoso and Williams, 2001). The stem cell population may migrate from the marrow during epithelial repair or may be present in the lung from the stage of embryogenesis. Attempts have been made to identify these cells in female sex-mismatched bone marrow transplant recipients, by assessing percentage of epithelial cells with Y-chromosomes. However no such population of epithelial cells was found (Davies et al., 2002).

The relevance of the stem cell is that potentially lifelong gene correction would be possible, but that a topical delivery approach is less likely to target this population of cells.

1.4.5 Gene Therapy for CF

The identification of the CFTR gene in 1989 has made gene therapy for CF possible. Within 4 years of cloning of the gene, the first gene therapy trials were completed. Since then, there have been 12 human trials of gene transfer agents. At present the

largest limiting factor appears to be low levels of delivery of GT agents (especially non-viral GT agents) and therefore presumably little production of CFTR protein. At present, there are no techniques to quantify CFTR protein. This is important because once the barrier of delivery is overcome, the potential toxicity of overexpression of CFTR will need to be investigated.

Gene therapy vectors fall into two categories – viral and non-viral gene transfer agents. This thesis is linked to the work of the UK CF Gene Therapy Consortium, whose focus is on non-viral gene transfer with the objective of developing a well-tolerated practical protocol that achieves clinical benefit upon repeated application.

1.4.5.1 Gene Therapy Vectors

A gene therapy vector is any agent that transfers nucleic acid in a functional form to the appropriate cell type.

1.4.5.1.1 Naked DNA

Naked DNA can transfect cells however, it is not stable by nebulised delivery methods, and could be degraded by endogenous or exogenous DNase in the ASL. If these hurdles could be overcome, it is an attractive candidate as it is easily formulated and manufactured.

1.4.5.1.2 Non-viral gene therapy vectors

1.4.5.1.2.1 Cationic Liposomes

Cationic liposomes are positively charged and are attracted to the negatively charged surface membrane of the cell. They can be complexed with DNA and cause compaction of the DNA. Liposomes can be monovalent or polyvalent. Examples used by the UKCFGTC include DOTAP, lipofectin, lipofectamine, PEI and GL-67. The mode of entry to the cell is most likely to be via the endosome, and release from the endosome is a significant barrier to these GTAs (Zabner et al., 1995, Zhou and Huang, 1994).

1.4.5.1.2.2 Compacted DNA

Compacted DNA, otherwise known as nanoparticles could assist gene transfer, as nanometre sizes would allow diffusion in to the nucleus. DNA can be compacted using

polyethylene glycol-substituted poly-L-lysine, forming unimolecular DNA particles (nanoparticles) (Ziady et al., 2003a, Ziady et al., 2003b, Ziady et al., 2002).

1.4.5.1.3 Viral gene therapy vectors

1.4.5.1.3.1 Adenovirus.

Adenovirus is the most commonly used viral gene transfer agent for CF gene therapy. Its mode of action is the best characterised of the gene therapy vectors. Adenovirus is a double-stranded linear DNA virus that possesses a natural tropism for respiratory cells and has the ability to transfect non-dividing cells. As viral DNA can integrate into the host genome (especially during cell division), there is an association with neoplasia (e.g. by insertion of viral promoters in front of human proto-oncogenes).

1.4.5.1.3.2 Adeno-associated Virus

Adeno-associated virus (AAV) is a modified parvovirus and delivered in CF GT trials to adults and children in the US (Moss et al., 2004). AAV offers good transfection efficiency and limited immunogenicity, whilst its ability to integrate into host genome has been reduced compared with adenovirus. Indeed it has been reported that human CF GT lung studies with AAV2 have shown a reduction in interleukin 8 - suggesting partial correction of the inflammatory status of the CF subjects airways. There is no convincing evidence of success of delivery after repeated dosing, and neutralising antibodies have been detected in BAL after AAV delivery. The neutralising antibodies may inactivate AAV, or may, after repeated dosing cause an immune response leading to inflammation and toxicity. Loss of efficacy after repeated dosing has been suggested with rises in interleukin 8 levels to initial levels within 3 doses (Moss et al., 2004).

1.4.5.1.3.3 Sendai Virus

Sendai virus (SeV) belongs to the paramyxoviridae family and the mouse is its natural host. SeV efficiently transfects human epithelia, through multiple mechanisms. Attachment and cytoplasmic entry is mediated by two envelope glycoproteins, haemagglutinin-neuraminidase (HN) and fusion protein (F) which interact with cholesterol and sialic acid respectively. This mode of transfection is very efficient.

However these proteins are markedly immunogenic, and repeated delivery of SeV has not been possible due to neutralising antibodies.

1.4.5.2 Target Transfection efficiency

Cystic Fibrosis is an autosomal recessive disease. Since heterozygotes, who have 50% of WT CFTR protein, do not develop CF lung disease, transfection of 50% of cells may not be necessary.

Evidence from multiple sources has led to the proposal that the required transfection efficiency needed may be as low as 5% of cells. Mixing experiments in cell lines showed correction of the electrical defect by adding as few as 6-10% corrected cells to an epithelial sheet (Johnson et al., 1992). Subjects with mild CF mutations such as R117H, retaining only 2% of estimated activity, are pancreatic sufficient and have mild lung disease, suggesting that even low rates of gene transfer may confer some protection against CF lung disease.

Intercrossing of CF and non-CF mutant mice is possible and leads to modulation of expression of CFTR. 5% of normal allele expression revealed a disproportionately large correction of the chloride ion transport defect (50% of normal) and essentially complete rescue of the intestinal disease (100% survival) (Dorin et al., 1996). Therefore it follows that even modest levels of transgene expression and only partial correction of CFTR channel activity may have a significant clinical impact.

Transfection efficiency of 5% is an achievable endpoint using viral GT agents, and maybe achievable using non-viral GT agents coupled with repeated delivery.

1.4.6 United Kingdom Cystic Fibrosis Gene Therapy Consortium.

The UK CF Gene Therapy Consortium combines the efforts of three centres within the UK with a wealth of experience and a long-term commitment to developing gene therapy - Oxford, Imperial College, London and Edinburgh. It has the specific aim of developing effective gene therapy for delivery to patients. The CF Trust, UK, supports the work of the UK CF Gene Therapy Consortium (UKCFGTC). Original and additional support comes from the Medical Research Council, Wellcome Trust, Chief Scientists Office, Scotland and others.

The gene therapy products developed will need to demonstrate gene transfer, protein production, functional correction, and efficacy and have the ability to be delivered repeatably for the lifetime of the CF subjects. The work of this thesis is to develop and validate assays that would reflect improvements in CF inflammation in the lungs of stable CF subjects and to develop surrogate markers of CFTR function.

The website for the UK gene therapy consortium is <http://www.cfgenetherapy.org.uk/> .

Non-invasive assays are advantageous since they avoid the problems of invasive procedures like bronchoscopy. Such problems include lack of repeatability and safety issues in subjects with respiratory failure. It is hoped that non-invasive assays would be better tolerated, enabling repeated testing of individual subjects. Therefore data could be generated in a longitudinal manner e.g. throughout cycles of infective exacerbation, or at multiple times in the weeks and months following gene therapy deliver.

Established assays of chloride efflux such as nasal potential difference (PD) require electrical contact with epithelium, therefore non-invasive techniques will not be able to assess chloride efflux directly (as would be possible using a bronchoscope). Instead of direct measure of changes in PD, the intention is to use surrogate markers of CFTR function. In addition, we anticipate assay of cytokines and chemokines will be useful to report on the inflammatory state of each individual.

1.4.6.1 Trial Design to optimise detection of Gene Transfer

It would be difficult for Gene Therapy studies commencing in children to be approved by the UK Gene Therapy Advisory Committee, who are the government body to advise on the ethical acceptability of gene therapy trials. They have evolved the ethical principle that children can be studied if only there is no possibility of relevant study in adults and if they, as individuals, are likely to benefit. This leads to difficulties as children are likely to be the group who respond best to gene therapy in that they would not have the high bacterial loads and severity of established lung damage that the adult group would have. Therefore, it is necessary that the adult group must first be studied. Although safety not efficacy is the sole objective in a phase 1 trial, to ensure approval of Phase 2/3 studies in children it is likely that efficacy as well as safety will have to be demonstrated in adults. Adults will have more severe lung disease, as by nature it is

progressive. They are more likely to have the characteristic thick tenacious sputum present when bronchiectasis is present. This tenacious sputum has long been known to reduce gene transfer efficiency. Consequently, adults recruited for GT trials are less likely to show demonstrable efficacy of gene transfer and therefore to gain clinical benefit. As gene transfer will not reverse bronchiectatic pathology (i.e. inflammatory pathology), markers of CFTR function are necessary alongside markers of inflammation. Steps to improve study design and increase likelihood of demonstrating efficacy would be to use patients with milder phenotype (e.g. FEV₁ 50-80%) and have a scaled introduction for older children once initial safety has been proven e.g. after first 6 months, recruit 12-16 year old to the study.

1.4.6.2 Assays of inflammation for Gene Therapy trials

At present, the optimal gene therapy vectors are being developed concurrently with the development of assays of efficacy. This makes both the assessment of different vectors and the assessment of assays of efficacy difficult. Assays that evaluate the inflammation of CF lung disease are necessary, as the inflammatory response in CF appears upregulated. It is anticipated that the inflammatory response will be downregulated with gene transfer. Proofs of principle of this theory have been demonstrated in both non-viral and viral gene therapy trials with reduction in interleukin 8 levels in airway surface liquid (Alton et al., 1999). Evaluation of novel inflammatory mediators would also be helpful, as there is overlap between CF and non-CF interleukin 8 levels. Novel biomarkers demonstrating a clear separation of CF and non-CF groups would improve likelihood of GT efficacy being detected.

Electrical measurements provide a functional end-point assay. However, it remains unclear how the electrical defect is related to disease pathology. Therefore, it may be more relevant to have assays that correlate better with disease processes and pathophysiology. For example, assays that correlate with lung function, reflect mucociliary clearance, inflammation and bacterial adherence may be more suitable to evaluate gene transfer efficacy.

1.5 Non-invasive sampling techniques in lung disease

Non-invasive sampling techniques to investigate airway inflammation are those which collect airway surface liquid (ASL), avoiding the sedation and/or anaesthesia required for bronchoscopy and bronchoalveolar lavage. Sampling ASL (using invasive techniques) is performed routinely during the clinical care of patients with respiratory problems and is known to be useful in monitoring severity and progress of many diseases (Nicolai, 2001). It can be performed by fiberoptic bronchoscopy (FOB), with either sedation and local anaesthesia or a general anaesthetic. Bronchoalveolar lavage (BAL) sampling can take place using this invasive sampling technique, and is generally performed alongside bronchial brushings and biopsies. This technique is reasonably well tolerated in children but is rarely used in the majority of CF adults, as bronchoscopy should not be performed in subjects with respiratory failure. The two non-invasive techniques investigated in this thesis are the collection and analysis of Exhaled Breath Condensate and Induced Sputum.

The potential to sample ASL without using an invasive procedure such as FOB is attractive in that it would allow a wider range of patients to be investigated e.g. those with respiratory failure. The EBC and Induced Sputum collection technique offers the potential to be safe, simple to perform and repeatable in each individual (Table 3). If successful, these techniques would allow ASL analysis to be applied on a large scale and longitudinally to clinical studies of CF .

	Bronchoalveolar Lavage	Exhaled Breath Condensate	Induced Sputum
Time	40 minutes including bronchoscopy	5 minutes	30 minutes
Safety	Unsafe in respiratory failure	Safe	Can cause wheeze and ↓ FEV ₁
Simplicity	No	Yes	Yes
Easily repeatable	No	Yes – within minutes	Yes - After 24 hours
Reproducibility	Yes	Not known	Not known

Table 3. Comparison of three techniques to sample ASL

1.5.1 Exhaled Breath Condensate

The potential of Exhaled Breath Condensate to be used as a research tool was first realised by Russian researchers (Iakovleva et al., 1987, Kurik et al., 1987, Sidorenko et al., 1980). This original work focused on the viscosity of “exhaled airway humors”. Independently, it was found that more than 200 compounds were present in exhaled air (Manolis, 1983) and exhaled breath condensate (Scheideler et al., 1993). Thereafter, groups started to investigate EBC principally to investigate changes to pulmonary surfactants, but also for other compounds such as acetylcholine, serotonin, and histamine (Goncharova et al., 1989). Such compounds, being non volatile in nature (albeit at very low concentrations) imply that EBC is comprised of more than just condensed volatile gases. This important observation has fueled further research in this field, as EBC may therefore contain components of Airway Surface Liquid (ASL) (Scheideler et al., 1993).

Initial research using EBC focused on the common respiratory disease, asthma (Goncharova et al., 1989). Biological mediators have been investigated, with particular focus on chemokine and cytokine mediators and their metabolic end products (Khyshiktyev et al., 1994).

Markers of oxidative stress have generated interest as they are thought to reflect the oxidative burst in cells and hence inflammatory responses. Markers of nitrative stress, also reflect components of the inflammatory process.

Strong evidence of a non volatile component to EBC has been published. Detection of ion, protein and lipid components have taken place (Effros et al., 2002, MacGregor, 2004, Montuschi et al., 1999).

Mass spectrometry is a sensitive technique to analyse multiple compounds using their molecular weights. It was first used to analyse the lipid component of EBC in 1997 (Iatsenko et al., 1997, Jobsis et al., 1997).

With regard to cystic fibrosis, several markers of inflammation have been investigated, including the exhaled gas nitric oxide and EBC nitrite and hydrogen peroxide (Hacein-Bey-Abina et al., 2002, Ho et al., 1999, Ho et al., 1998a, Ho et al., 1998b). The work of

this thesis is an evolution of these studies, specifically applied to CF in the context of gene therapy.

1.5.2 Induced Sputum

Induced sputum has been established as a research tool evolved from collection and analysis of spontaneous sputum. As sputum contains fluids expectorated from the lung, it is likely to contain Airway Surface Liquid.

Induced sputum is a technique which was developed to diagnose *Pneumocystis carinii* pneumonia in immunocompromised patients and is used for this purpose routinely in hospital care (Pitchenik et al., 1986). The technique involves asking subjects to inhale hypertonic saline solution for a set period of time and then they are asked to expectorate sputum. This sputum is used for further analysis.

It is possible that induced sputum samples fluid from the airway at a point more distal to that of spontaneous sputum as the inhaled saline nebuliser delivers saline to the conducting and distal airways. Ultrasonic nebulisers offer a larger particle size (5.58 μ m) than standard “Jet” nebulisers (3 μ m). This has an advantage in that a higher proportion of the particles deposit in the conducting airways (i.e. the target region where we would like to sample ASL) compared to the alveolar region (Rau, 2002).

1.5.3 Induced Sputum collection technique

Induced sputum collection techniques have been standardized to a greater extent than EBC collection. It is established that ultrasonic nebulisers should be used, with percentage of saline and the duration of delivery being the only variable to be standardized. Investigation of induced sputum has taken place in COPD, asthma and lung cancer (Khajotia et al., 1991, Ordonez et al., 2003, Pin et al., 1992, Sterk, 1997, Suri et al., 2003, Tsoumakidou et al., 2003).

Investigation of sputum has taken place in cystic fibrosis research for many years, with sputum microbiology being a mainstay of clinical care. Induced sputum protocols have been developed and established as safe in CF, to obtain ASL from patients who are too young to expectorate spontaneously (De Boeck et al., 2000).

As induced sputum contains cells along with a liquid phase, a greater number of investigations can be performed on the sample. The cell phase can be used to assess the inflammatory cell infiltrate in the airway and the differential proportion of each type of inflammatory cell is a good discriminator between diseases and has been shown to be

useful in asthma and COPD. The liquid phase of induced sputum has also been the subject of interest, with raised interleukin 8 and total protein being found (Sagel et al., 2001).

The method for induced sputum used in this thesis is derived from those of the initial investigators (Pin et al., 1992) and adapted by others (Pizzichini et al., 1996). Other investigators have extensively analysed induced sputum for inflammatory mediators in subjects with Cystic Fibrosis and have revealed that these in particular have investigated the differential cell counts, protein levels and inflammatory cytokine interleukin 8 (Ordonez et al., 2003, Sagel et al., 2001, Sagel et al., 2002, Suri et al., 2003).

1.6 Markers of Inflammation in CF Lung Disease

The various markers of inflammation can be broken down into their chemical subcomponents. An understanding of the immune response is necessary to identify the relevance of the various potential markers of inflammation to CF lung disease.

1.6.1 Inflammatory response

The inflammatory response can be broken down into two main types – the innate and adaptive immune responses.

The innate immune response acts via pathogen recognition receptors (PRRs). Molecular patterns, which are highly conserved on large groups of microbes, are termed pathogen associated molecular patterns and are recognised by toll-like receptors (TLR). Examples include lipopolysaccharide (TLR2/4), lipoteichoic acid (TLR2) and bacterial unmethylated cytosine-guanosine dinucleotides in DNA (TLR9). PRRs are present on all antigen presenting cells, dendritic cells and macrophages. Once pathogens are recognised, these receptors induce Nuclear Factor Kappa B production, leading to transcription of a wide range of inflammatory and immune-response genes such as TNF alpha, interferon gamma, Interleukin 1 Beta and interleukin 6.

The adaptive (acquired) immune response is activated via antigen presenting cells - dendritic cells and macrophages in the lung. They signal recruitment of immature dendritic cells and macrophages to the lung. This response is amplified by the innate immune response and TNF alpha and Interleukin 1 beta production. Macrophages are recruited from the circulation and activated via TNF alpha, Interferon gamma and Interleukin 1 beta. They secrete chemokines such as interleukin 8 (neutrophil attractant) and RANTES (eosinophils attractant) inducing humoral immunity with neutrophil influx and immunoglobulin secretion.

1.6.1.1 Neutrophil response

Neutrophils are manufactured in the bone marrow, travel through the circulation and adhere to venular endothelium (including the capillary endothelium in the lung) awaiting a chemotactic signal – particularly interleukin 8, but also to leukotriene B4. They respond by diapedesis through the vascular wall, migrate to the site of

inflammation to phagocytose and destroy microbes and other foreign material.

Phagocytosis is signalled as part of both the innate and adaptive immune responses. The neutrophil membrane envelops microbes and other particles forming a membrane bound vacuole. Neutrophil cytoplasmic granules contain a variety of bacterial inactivating and killing proteins including myeloperoxidase, elastase, lactoferrin, lysozyme, proteinase 3, cathelicidins, gelatinases and defensins (Borregaard et al., 2001, Wagner and Roth, 2000).

Killing and digestion occurs by fusion of toxic granules to the vacuole, and generation of the enzyme NADPH oxidase. This enzyme passes electrons from NADPH in the cytoplasm to molecular oxygen within the vacuole. Superoxide is generated by this oxidative (or respiratory) burst. A chain reaction occurs leading to rapid consumption of molecular oxygen, and generation of a variety of reactive oxygen and nitrogen species. Superoxide is converted into hydrogen peroxide, which in turn reacts with myeloperoxidase to generate hypochlorous acid and chloramines, both potent disinfectants (Nauseef, 1999).

1.6.1.2 Apoptosis and necrosis in the CF airway

Apoptosis is programmed cell death with organised disassembly. Uncontrolled cell death is termed necrosis. Cell membrane integrity is lost, leading to lysis, release of cell contents and an inflammatory response. The macrophage detects cell lysis products and releases proinflammatory mediators such as TNF alpha, IL8 and thromboxane B2.

Apoptotic cells express phosphatidylserine receptors leading to ordered cell death and clearance. CF airway fluid cleaves the phosphatidylserine receptor leading to unprogrammed cell death, necrosis and exacerbation of the inflammatory response in the CF airway (Vandivier et al., 2002). Therefore, as cell necrosis occurs in the CF airway, various inflammatory mediators involved in the inflammatory processes may be released into the ASL of CF subjects.

1.6.2 Potential Biomarkers of inflammation to be investigated

1.6.2.1 Oxidative stress

The oxidative burst of the neutrophil is responsible for the generation of an excess of reactive oxygen species, necessary for bacterial killing. It has been established that oxidative stress occurs in Cystic Fibrosis and a correlation between the lung function parameter FEV₁ and serum levels of lipid hydroperoxides has been shown (Brown and Kelly, 1994, Brown et al., 1996). Detection of a variety of surrogate markers of oxidative stress such as carbon monoxide and 8-isoprostane is possible and will be investigated.

1.6.2.2 Nitratative stress

As end products of oxidative stress are generated by the oxidative burst of neutrophils, products of nitratative stress are generated by reactive nitrogen species i.e. Nitric Oxide, peroxy nitrite, Nitrite and Nitrotyrosine. NO is generated in two ways.

- Inducible nitric oxide synthetase (NOS 2) expression can be induced by proinflammatory cytokines (Latzin et al., 2002). However this induction may not occur in CF as iNOS is less abundant by immunohistochemical detection in CF lungs compared to other respiratory pathologies (Meng et al., 1998). This reduction in iNOS in CF is confirmed to be at the RNA level in epithelium (Zheng et al., 2004). In addition, using FABP mice, the iNOS reduction appears to be site specific (iNOS levels low where CFTR is not expressed in nasal epithelium, but higher in the gut where CFTR was expressed (Steagall et al., 2000, Widdicombe, 2000, Zheng et al., 2003). This important finding suggests that products of iNOS could be low in CF and that it may be useful to report on success of CFTR GT.
- Additionally, NO is released from glutathione nitrated products (s-nitrosothiols). The majority of NO released from the airway (70-90%) is as a product of s-nitrosothiol release (Stamler et al., 1992). Importantly glutathione is low in the adult CF airway (Roum et al., 1993, Zaman et al., 2001) and cell line studies suggest it may be secreted through the CFTR channel (Kogan et al., 2003).

1.6.2.3 Lipid mediators

The predominant lipid mediators available are those involved in the arachidonic acid pathways generating leukotrienes and prostaglandins (Baraldi et al., 2003, Montuschi and Barnes, 2002a).

1.6.2.4 Ions

Ion changes are potentially interesting as changes in Na^+ , Cl^- , K^+ , pH and ammonium may reflect the basic CFTR function defect, changes in acid/base status or changes in capillary leakiness due to inflammation.

1.6.2.5 Proteins

Many proteins are involved in the inflammatory response. These fall into two general categories – the chemokines/cytokines e.g. interleukins and the killing/defence proteins e.g. elastase and defensins.

1.6.3 Proteomics and Novel Protein discovery

The biomarkers discussed previously are known and have been used to investigate inflammatory diseases in previous studies. An alternative approach is to attempt to discover novel biomarkers of disease. Proteomics methodologies could be applied for this purpose.

Proteomics is the genome wide study of proteins. The completion of the working draft of the human genome sequence signalled the key point in the evolution of the field of proteomics (Lander et al., 2001). Since then, work on the predicted human proteome (identification of the protein “complement” of gene expression) has begun. The proteome is much more complex than the corresponding genome as around 500,000 proteins are generated from some 30,000 genes. Most genes are subject to differential splicing which is cell type specific. After translation, the majority of proteins are then modified e.g. by glycosylation, phosphorylation and cleavage. The correlation between mRNA expression and protein function is consequently weak.

Differences in protein expression between diseased and healthy subjects may vary greatly. The human proteome is a highly complex and dynamic protein network and is generated from a static genomic blueprint. The field of study has evolved in two ways. Gel electrophoresis techniques have been established for many years (the first available reference investigating human samples was in 1968 (Cudny and Wald, 1968), and more recently very sensitive Mass Spectrometry techniques have been established. These techniques offer highly sensitive detection of proteins, and when coupled to gel electrophoresis techniques and human proteomic databases, we can identify the majority of proteins of interest.

Characterisation of the Human Proteome has just begun, with the focus on serum, plasma and liver samples initially. There are large-scale collaborative plans to complete study of the proteome of all biological fluids. There are no immediate plans to investigate the Airway Surface Liquid.

The investigation of the proteome of ASL in normal control subjects and in Cystic Fibrosis subjects is of interest as it is the ASL where CF lung disease is exhibited and proteins are the downstream product of gene regulation. By investigating the proteome

of CF lung disease, we may find markers of inflammation, protein products of modifier genes as well as surrogate protein markers of CFTR function.

As exhaled breath condensate is a dilute biological fluid, the nanogram sensitivity of gel electrophoresis may not be enough to detect protein biomarkers of CF lung disease. A highly sensitive mass spectrometry technique is an alternative - Surface Enhanced Laser Desorption/Ionisation (SELDI). This technique offers attomolar sensitivity, generating semi quantitative data with the advantage of removing interfering compounds such as salts and DNA. Analytical software allows differential expression analysis and recognition of patterns of different proteins in each sample. These techniques will be discussed further in Chapter 6.

In this thesis, polyacrylamide gel electrophoresis and SELDI mass spectrometry are both applied in an attempt to detect and validate novel markers of inflammation in EBC and Induced Sputum.

1.7 Introduction - Summary

This introduction has outlined Cystic Fibrosis lung disease, its progression and the need for CF gene therapy. For clinical trials of CF GT, biomarkers of CFTR function and of inflammation are needed to assess successful delivery. Non-invasive sampling techniques such as EBC and Induced Sputum could report usefully on the inflammatory state of the CF airway and could potentially also report on surrogate markers of CFTR function.

The work of this thesis will focus on the development and validation of such biomarkers for use in future gene therapy trials.

2.0 SAMPLE COLLECTION AND PREPARATION

2.1 The Ideal Marker

2.1.1 Hypothesis

Markers of inflammation for Cystic Fibrosis Lung Disease are measurable in samples collected non-invasively, and can be developed into clinically useful assays.

These assays would have the ability to reflect the level of

1. Inflammation in the CF lungs
2. CFTR function, in the context of gene transfer

2.1.2 The Optimal Biological Sample

2.1.2.1 The optimal biological sample would be

1. Repeatable in each subject
2. A sample the Airway Surface Liquid
3. Non – invasive
4. Well tolerated
5. Simple to collect and to analyse

2.1.3 The non-invasive samples assessed in this thesis were

1. Exhaled Breath Condensate
2. Induced Sputum

Where possible these samples were compared to the “gold standard” ASL sampling technique – fiberoptic bronchoscopy and the collection of bronchoalveolar lavage fluid. Cellular analysis of the nasal epithelium was used to validate markers identified.

Sampling constraints have meant that collection was limited for multiple reasons. The simplest collection technique – exhaled breath condensate, was collected locally and assessed with the majority of assays (in adults at the Western General Hospital, Edinburgh). Collaboration with other research groups within and without the UK CF Gene Therapy Consortium yielded further samples particularly with regard to paediatric EBC samples from Dr Steve Cunningham (Royal Hospital for Sick Children Hospital,

Edinburgh) and bronchoalveolar lavage fluid (Dr Tom Hilliard, Brompton Hospital, London). Induced sputum was collected locally in adults at the Western General Hospital, Edinburgh.

2.1.4 The Ideal Biomarker

The ideal marker would show clear separation between groups and be both repeatable and reproducible. Such a marker would be both highly sensitive and specific for the disease being assessed.

2.1.4.1 Rationale of biomarkers studied

To find biomarkers, a dual approach was taken. Firstly, established markers of lung inflammation were assessed to see if they were useful in assessing inflammation in the Cystic Fibrosis lung. These markers were identified in the scientific press as being useful in assessment of inflammation in other respiratory diseases including COPD, asthma and bronchiectasis. Secondly, an unbiased search for potential protein biomarkers using mass spectrometry was performed in the hope that novel markers of inflammation and of CFTR function could be identified.

We anticipated that individual biomarkers might not meet all of the required criteria, but by combining a “battery” of the best markers, the general hypothesis would be proven and a practical multimarker assay developed. Combining different tests requires multivariate statistical analysis techniques, and these were applied where possible throughout the thesis.

2.1.5 Subjects

The majority of patients recruited were adults attending the Adult Cystic Fibrosis Service in Edinburgh. Children attending the Paediatric CF clinic were also recruited where possible. Control subjects were recruited from staff at the Adult Centre and children attending the fracture clinic in the Paediatric Centre.

CF patients were assessed as “stable” (usual clinical condition, with no change in symptoms, lung function or sputum production) or “exacerbation” (combination of 3 or more of the following plus a clinical decision to treat with antibiotics).

1. Increased cough.
2. Increased sputum production.
3. Increased breathlessness.
4. Increased white count.
5. Decreased lung function (FEV₁).
6. Reduced exercise tolerance.
7. New pyrexia.

Written informed consent was obtained from Adults in the study and from the parents of children recruited. Verbal consent was obtained from each child. All studies were approved by the Lothian NHS Research Ethics Committee.

The subjects were recruited over a three-year period. Due to the limitations of small sample volumes of EBC, it was not possible to perform multiple assays in each individual sample. Most assays were validated initially with adult control samples, then adult CF samples and for the most robust assays samples from children were also used. An advantage of using both adult’s and children’s samples is that as CF lung inflammation is progressive, the levels of inflammation are more severe in the adult population. By comparing children’s samples with adult samples, we could gain more information about how the potential markers may relate to the development of lung disease.

2.2 Sample collection techniques

2.2.1 Exhaled Breath Gas analysis

Exhaled Breath Gas sampling techniques to assay exhaled carbon monoxide (CO) and nitric oxide (NO) have been established and validated. The techniques used in this thesis are as published in Thorax by my predecessor in the unit, Dr Ling Pei Ho (Ho et al., 1998a). A Logan Sinclair Research (Model LR2000; Logan Research, Rochester, UK) NO and CO analyser was used. It contains a combination of an electrochemical cell and chemiluminescence sensors. Subjects were asked to exhale through a mouthpiece, at expiratory flow rate of 50 ml per second for one complete breath (vital capacity). The plateau value read at the end of expiration was taken and the procedure was performed in triplicate and the mean +/- SD of the three values was used.

2.2.2 Exhaled Breath Condensate collection

2.2.2.1 Introduction

Exhaled Breath Condensate research is an evolving field and established and validated methods for collection at time of this research had not been published. Since the period of research was completed guidelines have been published jointly by the European Respiratory Society and the American Thoracic Society (Horvath et al., 2005). These guidelines represent a consensus view on optimal collection techniques, and are the same as used in this thesis. As yet, EBC is being used primarily as a research tool and has not been established in the clinical management of patients. It is hoped that in the future EBC sampling and the use of established assays would be used in clinical practice. Therefore, robust and repeatable sampling methods need to be established.

Exhaled breath condensate could conceivably contain compounds from anywhere in the respiratory tract from the mouth to the alveoli. There is a large water component, as evidenced by the low concentration of molecules assayed compared to serum, and also potentially dissolved volatile compounds and microdroplets of airway surface liquid.

Components of EBC which may be useful for biomarker analysis are:-

- Dissolved volatile compounds from the respiratory tract
- Microdroplets of Airway Surface Lining Fluid

2.2.3 EBC Collection Devices

2.2.3.1 EBC Collection technique

The exhalation of the different components may vary with respiratory maneuvers particularly with changes in respiratory flow rate. As the concentration of “solutes” in EBC is undoubtedly low, any techniques to increase microdroplet concentration would be useful.

2.2.3.2 Variables affecting EBC solute concentration

Exhaled breath condensate is collected by exhaling over a cooled surface. The potential variables in design of EBC collection device are of

1. Variation in condensing surface area.
2. Variation in condensing surface temperature.
3. Variation in condensing surface chemistry.
4. Alteration in respiratory pattern of exhalation.
 - Respiratory flow
 - Respiratory volume (i.e. minute ventilation)

2.2.3.3 Exhaled Breath Condensate – Methods

Two techniques were used for collection of EBC. The first, using PFA tubing in ice was used at commencement of this work, and later a commercially available system, Ecoscreen from Jaeger-Toennies (Jaeger-Toennies, Germany) was used.

2.2.3.4 PFA tubing Sample Collection technique

Perfluoroalkane (PFA) tubing was submerged in ice at approximately -4°C. Tubing dimensions were 4mm diameter, 1.5m long. The narrow bore of the tubing was chosen to facilitate closure of the soft palate, reducing nasopharyngeal contamination of EBC.

The tubing was cooled for at least 15 minutes in the ice, before use. Patients were asked to rinse their mouths with tap water and then asked to exhale through the tube for approximately 5 minutes (figure 10). The sample was collected into a Universal container at the other end of the tube. After 5 minutes of exhalation through the tube, approximately 1 ml of Exhaled Breath Condensate (EBC) was collected.

2.2.3.4.1 Disinfecting Teflon Perfluoroalkoxy (PFA) Tube

Some of the biomarkers of interest were products of reactive species generated by the respiratory burst. The disinfection agents used to clean EBC collection equipment contain reactive species such as reactive chlorine and reactive oxygen species.

Therefore, it was important to remove the disinfecting agents thoroughly after they have contacted the equipment, to avoid false oxidation and nitration of EBC samples.

The technique used Virkon, a chlorine based disinfecting solution (Antec, USA). PFA tubing was submerged for 30 minutes, then flushed through with warm tap water for 10 minutes and finally flushed with distilled water. For drying, high flow oxygen was piped through the tubing to flush out any water. Both ends of the tubing were capped with parafilm to reduce contamination before use.

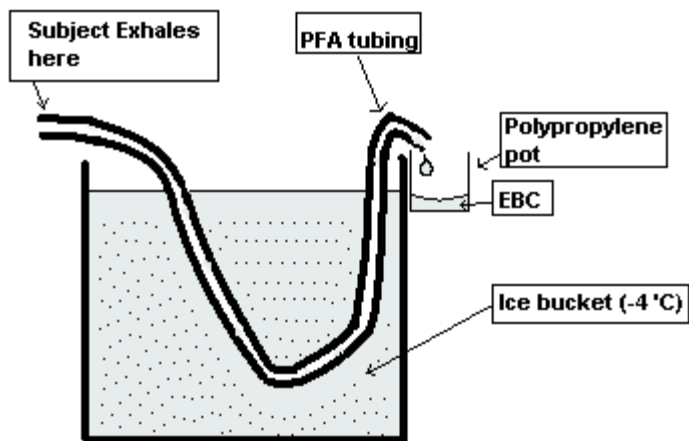


Figure 10. PFA tubing apparatus

The PFA tubing was submerged in ice and the exhaled breath condensate was collected into a polypropylene pot.



Figure 11. Patient using PFA tubing apparatus.

This figure demonstrates a patient exhaling through the PFA tubing apparatus.

2.2.4 Jaeger Toennies Ecoscreen EBC collection device

A commercially available collection device, the Ecoscreen (Jaeger-Toennies, Germany) was investigated when it became commercially available in 2001.

Using a system of valves, patients are able to exhale through a metal condensing chamber kept at -20°C. The valve system allows the patient to breathe tidally without having to remove their mouth from the mouth piece.

2.2.4.1 Jaeger Sample Collection Technique

This system comprises a mouthpiece and valve system attached to a cooled metal alloy sleeve. A polypropylene pot collects EBC, and a saliva trap collects saliva to prevent contamination of the EBC (Figure 11, 12 and 13).

The mouthpiece/valve manifold was inserted into cooling unit and allowed to chill for a minimum of 15 minutes. Patients were asked to rinse their mouths with water and then use nose clips. Patients were then asked to breathe “normally” through the mouthpiece for 5 minutes i.e. tidal breathing. Once sample collection was complete, the metal condensing chamber and polypropylene pot were spun for 30 seconds at 2000rpm to minimise sample loss in the alloy sleeve. The sample was then transported on ice to the laboratory for analysis and/or storage.

2.2.4.2 Disinfection of the Jaeger Ecoscreen collection device

Removal of disinfection agents was performed by flushing with water afterwards. The technique used was to use a dilute Virkon solution. The mouthpiece and collecting system was then soaked for 20 mins before rinsing with warm water tap and flushed for 2 hours. Afterwards the entire system was flushed with distilled water, air dried and then stored in a clean sealable bag until use.

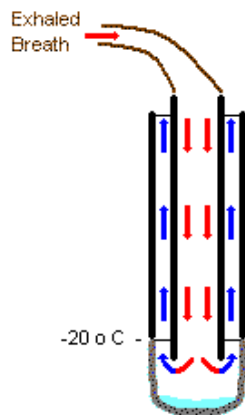


Figure 12. Diagrammatic representation of cooling system from Jaeger device.

The direction of exhaled breath through the breathing tube is indicated. It is cooled on metal alloy (shaded black). As a result of cooling, the exhaled breath condenses and is collected in a polypropylene container (shaded grey).



Figure 12. Jaeger Mouthpiece and EBC collection chamber and subject breathing through device.

Subject breathing through Jaeger collection device. Note noseclips are used and subject is breathing tidally.

2.2.5 Which sampling technique to use

When the Jaeger EcoScreen was initially used on CF subjects, it was clear that they preferred breathing tidally to the Teflon tubing (forced exhalation) method and commented that it was far less tiring.

As we were primarily interested in collecting microdroplets of ASL, we compared the EcoScreen with the Teflon tubing method (in healthy control subjects) and found that total protein concentrations using the EcoScreen were reproducible, and within a tight range (0.5 – 25.5micrograms/ml). We found the majority of sample collected using the Teflon tubing to be below the level of detection of the assay (0.5micrograms/ml) and no sample gave a figure as high as the median EcoScreen level of total protein. Therefore, we chose to use the EcoScreen due to patient preference and enhanced detection of ASL microdroplets.

2.3 Induced Sputum Collection

The technique of collecting of Induced Sputum has been established and validated (Pin et al., 1992, Pizzichini et al., 1996). I am grateful to Deborah Parker and Ian Pavord of the Department of Respiratory Medicine and Thoracic Surgery, Glenfield Hospital, Leicester for teaching me the clinical and laboratory techniques required for induced sputum collection and analysis. These techniques are derived from those used in the clinic at Glenfield hospital.

2.3.1 Induced sputum collection technique

The nebuliser used was a Devilbiss Ultra-Neb large volume ultrasonic nebuliser (Devilbiss, USA) and the nebuliser solution used was 3% (hypertonic) saline (Tayside Pharmaceuticals, UK).

Initially a saliva sample was collected into a “universal” container. Baseline spirometry was recorded – FEV₁ and FVC. Then 5mg of Salbutamol was administered. Spirometry was then repeated.

Patients were asked to blow their nose and rinse their mouth with water before proceeding with the 3% saline inhalation. Patients were requested to breathe normally with the nebuliser mask in place. Any secretions coughed were collected into a sterile container, labelled for each inhalation period. At the end of each inhalation – 4 minutes, patients were instructed to blow their nose and rinse their mouth with water, and then be encouraged to cough to produce sputum. Then FEV₁ was again recorded. If FEV₁ fell by > 20% post nebulisation then the sputum induction process was discontinued. The inhalations were repeated 3 times to a total of 12 minutes. The collected sample was then processed within 2 hours of collection.

2.3.2 Induced Sputum Processing

The mucus plugs were separated from saliva by applying the induced sputum to a Petri dish which was then rotated to separate saliva from the mucus plugs. Then the plugs were removed using curved forceps and transferred to another Petri dish and mixed using curved forceps.

Mucus plugs were reduced using DTT by transferring the plugs to a preweighed 10 ml falcon tube. 0.1% DTT was added, vortexed for 15 seconds and then mixed on a sample rotator for 15 minutes. The fluid volume was doubled with Dulbeccos PBS.

The sample was filtered through pre-wetted nylon gauze (45µm). To separate the cellular and soluble components, the sample was centrifuged at 800g for 10 minutes (4°C). The supernatant was aspirated and stored at – 80°C until further analysis.

The cell pellet was resuspended to approximately 1×10^4 cells/ml. Cytospins were prepared (400 rpm for 5 minutes), and slides were fixed using methanol for 10 minutes. Haematoxylin and eosin staining for 10 minutes was performed at a later stage to assess differential cell counts.

2.4 Bronchoalveolar Lavage fluid collection

Bronchoalveolar lavage fluid was kindly supplied by Dr Tom Hilliard of the Royal Brompton Hospital as part of the UKGTC collaboration.

2.4.1 Subjects

Children, in whom, bronchoscopy was being performed for clinical reasons were recruited. BAL was performed in 34 children with CF and 30 non-CF children. Samples were collected from patients undergoing bronchoscopy as part of their routine respiratory care. Therefore the majority of the non-CF group had inflammatory lung conditions. The majority of subjects had recurrent lower respiratory tract infection (18) or primary ciliary Dyskinesia (6). Two subjects had cardiac disease, one under investigation for haemoptysis and one had left lower lobe bronchiectasis.

Ethical approval for these studies was granted by Royal Brompton & Harefield NHS Trust and National Heart and Lung Institute Ethics Committee, and the Riverside Research Ethics Committee.

2.4.2 BAL processing technique

Bronchoscopy was performed under general anaesthesia. The fiberoptic bronchoscope was wedged in the middle lobe. 8 x 30ml aliquots of 37°C normal saline were instilled and then immediately aspirated. The aspirated (lavage) sample was put onto ice. The sample was filtered through gauze to remove debris. The cells were spun off (10 mins at 4°C – 1000 rpm). The supernatant was stored at -80°C in polypropylene eppendorfs.

2.4.3 BAL TCA precipitation technique for 2D gels

For polyacrylamide gel electrophoresis, it was necessary to remove interfering compounds such as salts from the sample. Trichloroacetic acid precipitation was used for this. One ml of BAL fluid was added to an eppendorf. 30% trichloroacetic acid (TCA) in acetone containing 60mM DTT was added and left at room temperature for 1 hour. The sample was spun at 13000rpm for 15 minutes. The supernatant was discarded and the remaining pellet with cold acetone containing 20 mM DTT. Again the sample was spun at 13000 rpm for 5 minutes and washed with acetone/DTT (repeated twice). The sample was air dried - by incubating at 37°C for 10 minutes. The

sample pellet was resuspended by adding a small aliquot of ultrapure water and SDS sample buffer.

2.5 Freeze Drying

Freeze drying was necessary in various part of the thesis due to low concentrations of solutes in EBC. Freeze-drying is the process of removing water from frozen material through sublimation. Ice sublimates directly to vapour when the pressure is lower than 6.11 mbars. We used a Christ Alpha (Christ, Germany) freeze dryer that operates under partial vacuum up to 0.25 mbars. This gives a minimum drying temperature of -34°C.

2.5.1 Pre-freezing

Pre-freezing of samples is necessary as the freeze drying technique rapidly cools the sample to -30°C and causes a vacuum within seconds. If the sample is not pre-frozen, trapped oxygen in the sample expands in the vacuum, causing the sample to bubble and spill. When samples are pre-frozen (30 minutes in -80°C freezer or 10 minutes in a dry ice/ethanol bath at atmospheric pressure), no loss of sample occurs.

2.5.2 Freeze Drying Pressure

As EBC is predominately water, its eutectic (freezing) point should be 0°C. The minimum temperature for drying is 10°C below the eutectic temperature, to avoid the sample melting and evaporating. The sample temperature is controlled by the vacuum. Therefore, the drying vacuum used was 2.560 mbars giving a drying temperature of -10°C.

2.5.3 Main Drying

Once the freeze drier reaches the appropriate temperature and pressure, the sample begins drying. This removes the majority of the water. It takes approximately 4 hours to dry the sample. Then the pressure is reduced for final drying to remove any remaining crystalline bound water for a further 30 minutes.

2.5.4 Freeze Drying Technique

The samples were checked every four hours until freeze drying was complete.

2.5.5 Calculation of percentage of sample reconstituted

Once the sample had been freeze-dried, very small quantities of powder were visible along the side and bottom of the well.

For assay purposes, the dry powder sample needs to be reconstituted in either ultrapure water or an assay specific buffer. For EBC analysis, samples were freeze dried from 1ml and reconstituted using 10 μ L of appropriate sample buffer. Reconstitution of such small volumes is difficult as some of the dry powder sample may fail to resolubilise back into solution.

2.5.6 Validation of Freeze Dying/Reconstituting as applied to EBC and Nitrite

40 mls of exhaled breath condensate was collected, by prolonged sampling using the Jaeger EBC system, from 3 healthy control subjects. This EBC was pooled and spiked with sodium nitrite at a known concentration (10 μ M). The spiked breath condensate was freeze dried in 40x 1ml aliquots and reconstituted in 100 μ L of ultrapure water. From the 40 samples processed, a return of 88% (range 86-91%) was achieved with nitrite.

Sample recovery was at a high level (88%) and is likely to represent the high water solubility of sodium nitrite as this is an ion. It should be considered that reconstitution of other compounds which are not as highly soluble such as proteins may not give as high a percentage of recovery and therefore losses in the reconstitution process should be anticipated to be higher than with sodium nitrite.

2.6 Statistical Analysis

2.6.1 Sigma Stat 2.03 and Sigma Plot 2001 (SPSS)

These statistical software packages allow analysis of data using standard statistical testing methods. As the majority of data generated were non-parametric, the statistical test used was Mann Whitney Rank Sum with Bonferroni correction for multiple analyses. Correlation coefficients were calculated using Pearson's correlation coefficient. Sigma stat was used to calculate linear regression equations and Microsoft Excel used to extrapolate such data. Graphical representation of the data was generated as box plots using sigma plot (Figure 15).

Data were expressed as median and as an interquartile range (IQR) i.e. 25th and 75th quartiles.

The further statistical software to analyse SELDI data will be discussed in chapter 6.

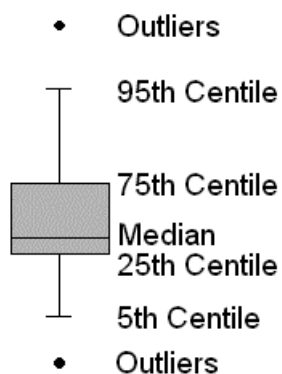


Figure 15. Box plot representation of data.

The majority of data generated in the studies were non-parametric. Therefore box plots were used to show median and interquartile ranges.

2.7 Bioinformatics tools used

2.7.1 Laboratory Protocols Online

This laboratory reference website is a reference tool to search for laboratory protocols.

<http://www.protocol-online.org/>

2.7.2 NCBI (National Centre for Biotechnology Information).

This website (<http://www.ncbi.nlm.nih.gov/Entrez>) was used to access many databases, particularly Pub Med. <http://www.ncbi.nlm.nih.gov/Entrez>

2.7.3 SWISS PROT and TrEMBL

Swiss-Prot is a “protein knowledgebase” and TrEMBL is a computer-annotated supplement to Swiss-Prot. This searchable database offers multiple proteomics tools on one website. It offers the proteomics sequences derived from the human genome project (along with other taxonomies). <http://us.expasy.org/sprot/>

2.7.4 ProFound

The Rockefeller University ProFound website contains a powerful search engine to assist with identification of proteins from the mass of their peptide digested fragments - <http://129.85.19.192/prowl-cgi/ProFound.exe>

2.7.5 MASCOT

This database was used to confirm the identity proteins from MALDI sequences generated by fragmented peptides.

http://www.matrixscience.com/search_form_select.html

2.7.6 Association of Biomolecular Research Facilities - Delta Mass Database

This website contains a concise database of posttranslational modifications and the mass shift effect they have on proteins.

<http://www.abrf.org/index.cfm/dm.home?AvgMass=all>

2.8 Assay Techniques – Chapters 3 - 6

Many of the assays applied in this thesis have been used and validated by others using samples such as plasma and serum.

Exhaled Breath Condensate is a dilute biological fluid when compared to serum and therefore assays need to be suitably sensitive to measure the compounds of interest.

As the assay techniques applied are varied, the methodology, results and discussion for each assay techniques have been combined and are detailed in the following chapters.

Chemical assays Chapter 3

- CO and NO
- Nitrite and Total Protein

Enzyme Immunoassays Chapter 4

- ELISA – Interleukin 8
- EIA – Leukotrienes, Isoprostane and Nitrotyrosine

Ion analysis Chapter 5

- pH
- Ion selective electrodes – Ammonium, Na⁺, Cl⁻ and K⁺

Proteomic analysis Chapter 6

- Polyacrylamide Gel Electrophoresis
- Surface Enhanced Laser Desorption/Ionization

3.0 – Chemical Assay Techniques

3.0 – Chemical Assay Techniques

3.1 Chemical assays

The chemical assays applied in this thesis are used to quantify the exhaled gases carbon monoxide and nitric oxide, the ion nitrite and total protein.

3.1.0 Exhaled Carbon Monoxide

3.1.1 Rationale

Exhaled carbon monoxide has been proposed as a marker of oxidative stress. The expression of the enzyme haemoxygenase can be induced by oxidative stress and as CO generation reflects haemoxygenase activity (Horvath et al., 2001), it may be used as a surrogate marker of oxidative stress activity in the lung. Haemoxygenase is the enzyme which catabolises haemoglobin (biliverdin) to bilirubin, releasing CO and iron in the process.

This marker has been established as being raised in stable Cystic Fibrosis subjects (Paredi et al., 1999). It has been shown to be raised further during infective exacerbation (Antuni et al., 2000).

3.1.2 Methods

A Logan Sinclair Research NO and CO analyser was used. It contains a combination of an electrochemical cell and chemiluminescence sensors, with the electrochemical cell measuring CO levels. Subjects were asked to exhale through a mouthpiece, at expiratory flow rate of 50 ml per second for one complete breath (vital capacity). Three replicates were performed in each subject and the average was used.

Subjects

The subjects included in this study were 15 healthy control subjects (adults) and 30 stable CF adults.

3.1.3 Results

There was no difference in CO between stable CF subjects (n=30, Median 4.2 (IQR 4.0-5.2) and non-CF subjects (n=15, Median 4.0 (IQR 3.5-6.1) p=0.754. See Figure 16.

3.1.4 Discussion

Exhaled CO is not different between stable CF subjects and non-CF subjects. Therefore, eCO cannot be used to discriminate between CF and non-CF individuals. Because of this, its role for gene therapy trials would be limited to reporting on significant toxicity.

It is not clear why these results were different from those previously reported. The median was similar to previously reported (Antuni et al., 2000, Paredi et al., 1999), but the range of data in this study was wider, particularly in the control group. One factor that could confound the study is that eCO is raised in smokers, therefore we were dependant on the study subjects reporting honestly about smoking habit. All subjects recruited denied smoking. However, two of the control subjects and one of the CF subjects had an eCO >10ppm, which was higher than would be anticipated in any non-smoker.

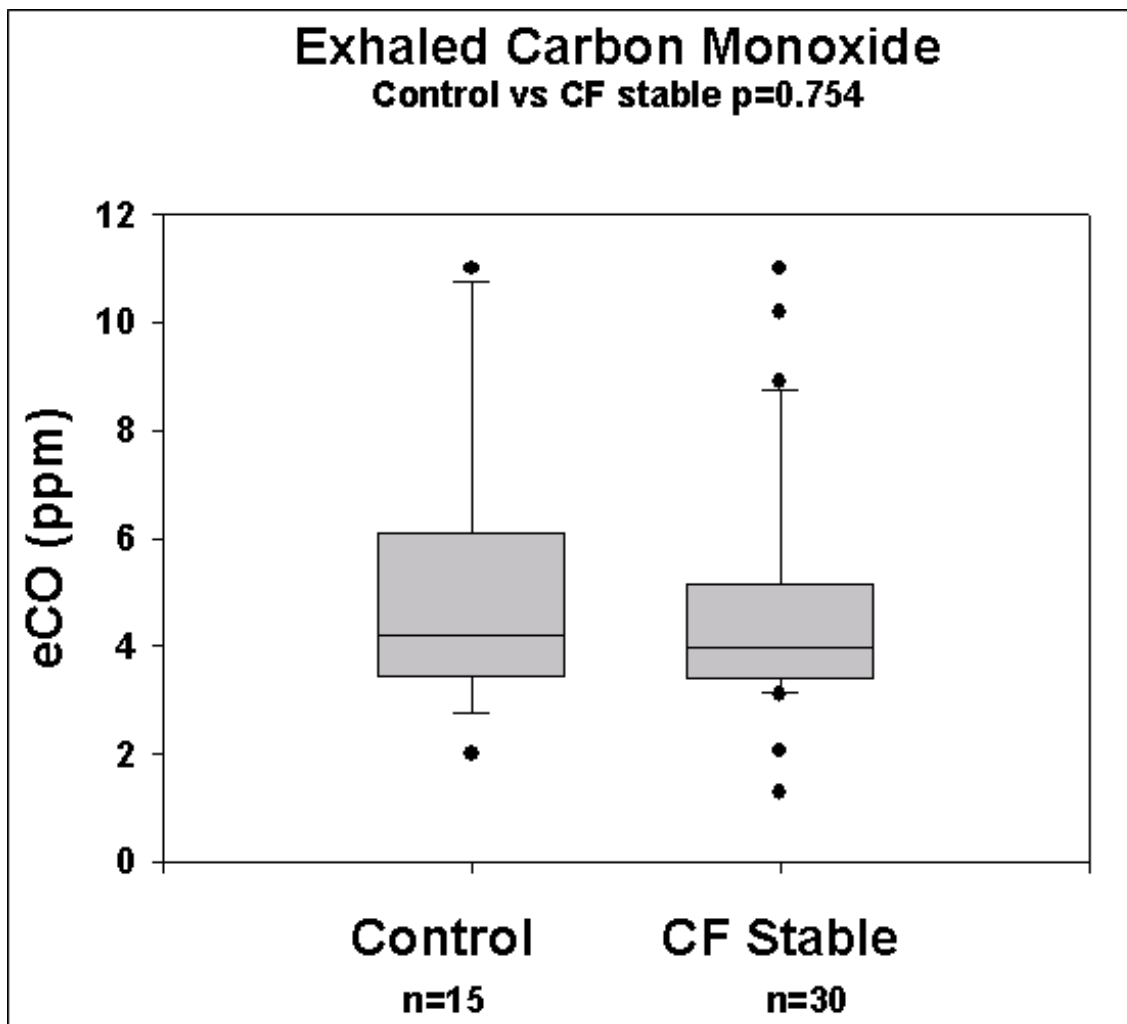


Figure 16. Exhaled CO levels in controls and CF.

Box plot of data in adults. Exhaled CO levels are not different between control and CF subjects.

3.2 Nitric Oxide

3.2.1 Rationale

Nitric Oxide is a gaseous product of nitrative stress. Its production could reflect reactive oxygen species generated in the tissues, inducible nitric oxide synthetase (NOS 2) activity (induced by proinflammatory cytokines and potentially by CFTR) or by release from glutathione nitrated products (s-nitrosothiols).

It is exhaled in the gas phase and therefore can be measured in exhaled breath. It has been shown to be no different between CF subjects than controls (Ho et al., 1998a, Ho et al., 2000). Indeed, some studies investigating children with CF have suggested that NO levels are lower in CF individuals, in particular of exhaled breath (Balfour-Lynn et al., 1996, Elphick et al., 2001, Grasemann et al., 1997) and nasal air (Dotsch et al., 1996).

3.2.2 Methods

As with eCO measurements, a Logan Sinclair Research NO and CO analyser was used, utilising the chemiluminescence analyser. Again, subjects were asked to exhale through a mouthpiece, at expiratory flow rate of 50 ml per second for one complete breath (vital capacity).

Subjects

The subjects included in this study were 15 healthy control subjects (adults) and 30 stable CF adults.

3.2.3 Results

Exhaled Nitric Oxide was not different between stable CF subjects (n=30, Median 2.1(IQR 1.8-3.4)) and controls (n=15, Median 2.7 (IQR 2.0-4.6)) p=0.214. The median CF level is lower than in the control group, at just above the 25th quartile of the control group (see Figure 17).

3.2.4 Discussion

There is no significant difference between the control and CF group data. Due to the level of inflammation in the CF subjects airways, even when stable, it would have been anticipated that they would have higher than normal levels of eNO.

The median exhaled NO was lower in Stable CF subjects than controls. These data are consistent with previous reports of lower NO measurements in Cystic Fibrosis children. The difference was not significant in adults due to the wider range of spread of data are seen in adult subjects.

In CF lung disease, the low median NO could be explained by the three following mechanisms.

1. iNOS production and activity is reduced in CF, via the Signal Transducers and Activators of Transcription pathway (STAT). A direct link between between iNOS and CFTR expression has been shown in cell line and mouse studies (Steagall et al., 2000, Zheng et al., 2004). In this study, levels of iNOS reflected CFTR activity in CFTR null and wild type mice. Where tissue specific promoters of CFTR were used (FABP), iNOS levels were normal in that tissue (intestinal tract). This raises the prospect that successful CFTR gene transfer may cause a rise in iNOS and eNO levels.
2. The majority of NO is released from s-nitrosothiols. BAL from adults with CF have demonstrated lower glutathione levels than control subjects (Roum et al., 1993). Whether this is a direct consequence of the CF mutation or due to infection and inflammation cannot be ascertained but it has been postulated that CFTR may enhance glutathione efflux..
3. eNO can be consumed by denitrifying bacteria, present in the airways of Cystic Fibrosis subjects. *Pseudomonas aureginosa* can metabolise nitric oxide through assimilatory and dissimilatory pathways (Gaston et al., 2002).

Although eNO in stable CF is not different from control subjects, the marker could still be useful in gene therapy trials as the levels could rise due to successful CFTR GT. Successful CFTR expression could cause an increase in iNOS expression and an increase in CFTR enhanced glutathione levels generating greater quantities on eNO.

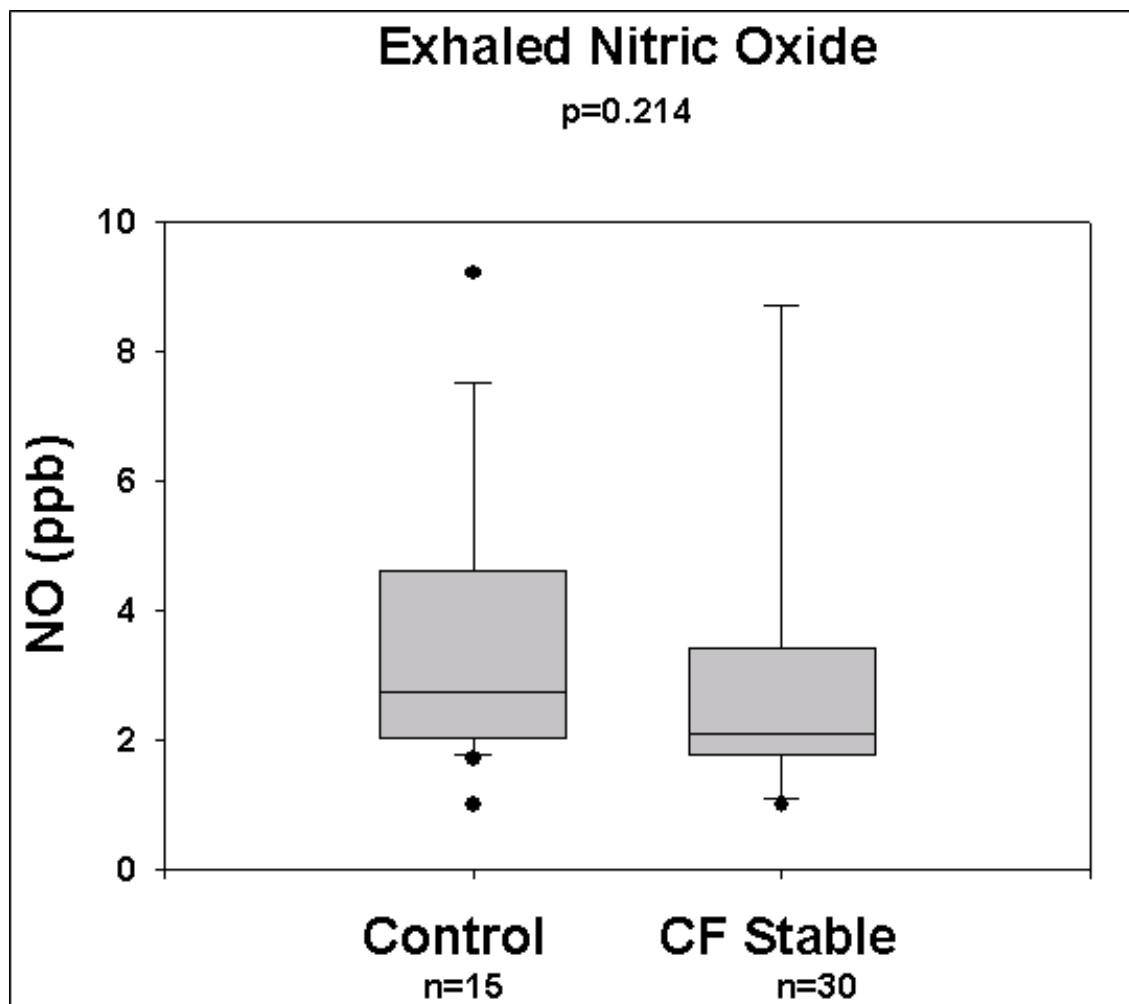


Figure 17. Exhaled NO levels in controls and CF stable subjects.

eNO levels were not different between control and CF subjects. The median eNO in the CF subjects can be seen to be lower than control subjects - at 25th quartile level of control subjects.

3.3 Nitrite

3.3.1 Introduction

Nitrite is a stable end product of NO metabolism (van der Vliet et al., 1999). This end product has previously been shown to be raised in CF (Ho et al., 1998b) and in CF children (Cunningham et al., 2000), and has been confirmed as being raised in patients with severe asthma, cystic fibrosis and COPD, but not in smokers or patients with mild asthma (Corradi et al., 2001).

3.3.2 Methods

The reaction used to quantify Nitrite (and nitrate) is the Greiss reaction and this has previously been modified and validated for use in EBC analysis in CF subjects. This protocol requires that the EBC sample be assayed as soon as possible after collection as decay in the nitrite signal has been reported to occur.

The reagents used were 2% sulphanilamide in 4% orthophosphoric Acid and 0.2%-1-Naphthyl-ethylenediamide dihydrochloride (NED), Sodium Nitrite standard solution 100mM. A standard curve was generated - 0.00, 0.5, 1, 2, 4, 6, 8, 10 μ M, and run in triplicate. Duplicate samples were used. 100 μ L of sample or standard was added per well. The sulphanilamide solution and NED were mixed in a Petri dish, and 25 μ L added to all wells. The microtitre plate was then stored in the dark for 15 minutes before reading plate at 540nm in a Spectrophotometre microtitre plate reader. Quantitative data were derived using sigma stat software and linear regression analysis.

To determine whether nitrite assays could be performed on stored samples of CF Exhaled Breath Condensate – 200 μ l aliquots were assayed immediately using Greiss reaction and others frozen at -80° C and stored for 7 days before assaying. There was no difference between samples assayed immediately and after 7 days (n=6, p=0.899).

Subjects

The subjects included in this study were 33 healthy control subjects (adults) and 29 stable CF adults.

3.3.3 Results - Nitrite

We found no difference in nitrite between stable CF adults (n=29, Median 3.43 (IQR 2.58-5.00)) and control subjects (n=33, Median 3.80 (IQR 2.00-5.59)), p=0.911. See Figure 18.

3.3.4 Discussion

There was no difference in nitrite between CF and control subjects. This result differs from previous published data which was generated using the same analysis techniques (greiss reaction) (Ho et al., 1998b). The only methodological difference was that our sampling technique used the Jaeger EcoScreen and tidal breathing rather than forced exhalations via PFA tubing. This may have influenced the outcome.

As nitrite is a stable end product of eNO metabolism, these nitrite data support the previous eNO data. The argument that successful CFTR gene therapy transfer could cause a rise in eNO levels therefore also applies to nitrite. The possible reasons are via reduced iNOS expression, reduced glutathione efflux or increase metabolism by *Pseudomonas aeruginosa*. As with eNO, successful correction of CFTR by gene therapy could lead to a rise in nitrite by inducing iNOS expression or by increasing glutathione efflux. As it is a simple and easy marker to measure, it should be included in the battery of non-invasive tests performed in GT trials.

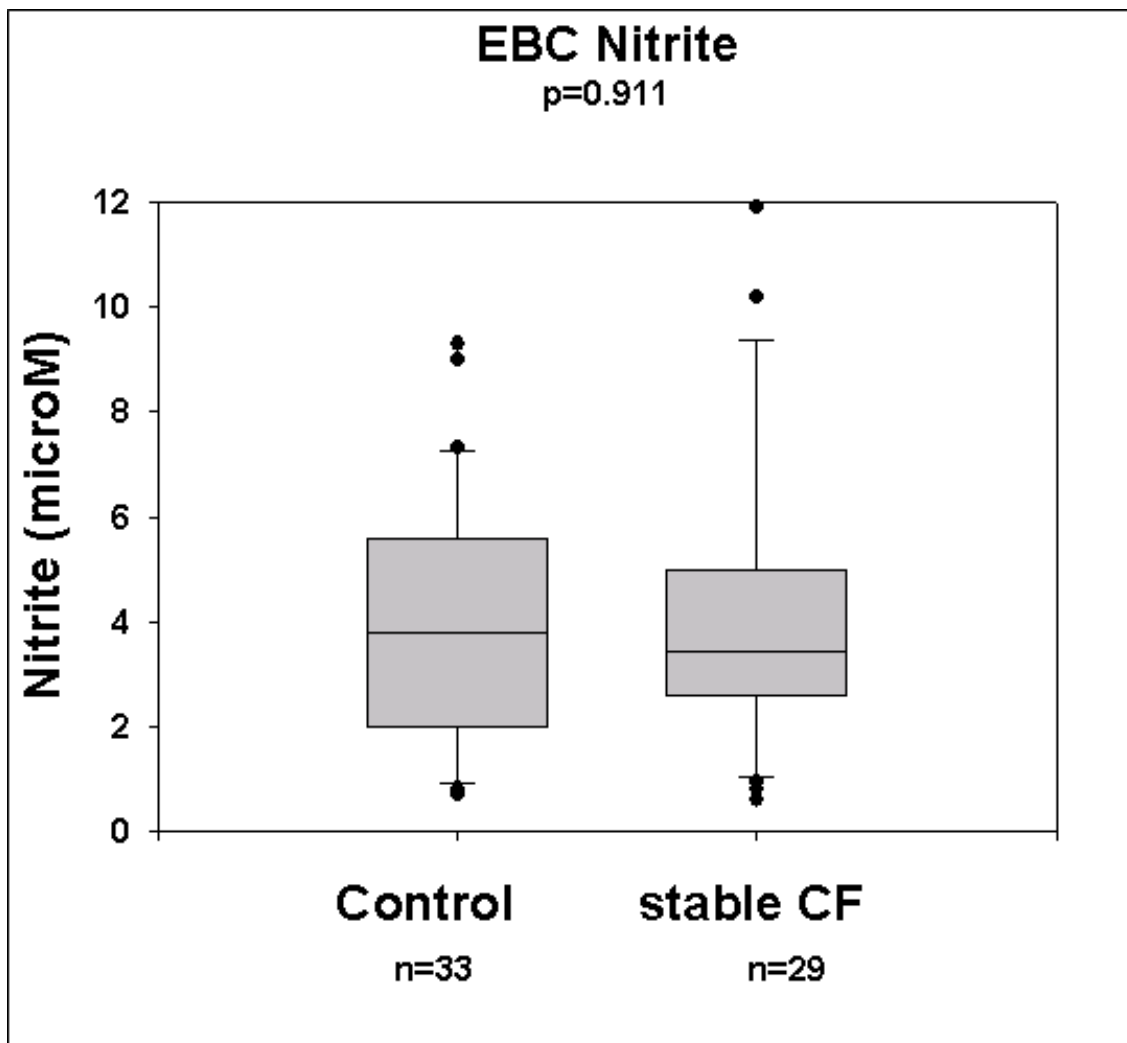


Figure 18. EBC Nitrite levels in controls and CF stable subjects.

EBC Nitrite levels are not different between control subjects and stable CF subjects.

3.4 Total Protein

3.4.0 Quantification of EBC protein concentration using bicinchoninic acid assay

3.4.1 Introduction

Total protein of EBC has been proposed as a reporter of inflammation as it was found to be raised in smokers compared to non smokers (Garey et al., 2004).

CF inflammation leads to an inflammatory infiltrate into the ASL, as well as capillary leak of serum proteins through loosening of the tight junctions between epithelial cells. These pathophysiological processes could lead to an increase in ASL (and therefore EBC) total protein concentrations.

Total Protein has also has been proposed as an internal standard against which other markers could be compared (Effros et al., 2002). This would allow interpretation of results, by correcting for variation in the number of microdroplets of ASL collected.

3.4.2 Methods

Previous publications in analysis of EBC have used the Pierce BCA assay to quantify Total Protein concentration (Effros et al., 2002). Using EBC collected from CF and Non CF subjects, the limit of detection of this assay was just above the levels in EBC (5µg/ml). Therefore, the microBCA technique was used to quantify proteins in this work as this was 10 fold more sensitive (lower limit of detection 0.5µg/ml).

The bicinchoninic acid (BCA) assay relies on the reaction of the peptide bonds between amino acids and copper. The reduction of copper leads to the formation of a purple complex with BCA. The microtitre plate assay was read on a spectrophotometer at 540nm. Interfering compounds can affect this assay (including H₂O₂, ascorbic acid, uric acid, lipids, and iron and reducing sugars).

A linear standard curve was generated from 0.5 to 200µg/ml using bovine serum albumin in ultrapure water, $r=0.999$. The reaction requires 150µL of EBC sample and is incubated for 2 hours in the BCA solution.

The standard curve also generated in acidified solutions at pH 4,5 and 7 (using hydrochloric acid) to ensure that differences in EBC pH would not have any effect on total protein detection (no pH effect was found).

Subjects

The subjects included in this study were 11 healthy control subjects (adults) and 9 stable CF adults.

3.4.3 Results

Total Protein ($\mu\text{g/ml}$) was raised in CF subjects ($n=9$, Median 16.15 (12.78-20.95)), compared to controls ($n=11$, Median 3.07 (11.08-13.40)), $p=0.027$. See Figure 19.

3.4.4 Discussion

Total protein was raised in CF subjects and therefore could be used as an end-point in future gene therapy trials.

The total protein concentrations detected in this study were within the wider range quoted in previous publications (Ballard et al., 1999, Effros et al., 2002, Scheideler et al., 1993, Simpson et al., 2005). The range of total protein in our sample group was narrower, but the reasons for this are not clear. One possibility is that our sampling technique to collect EBC reduced intersubject variation by using the Jaeger EcoScreen system using tidal breathing was used rather than using forced exhalations as with the PFA tubing technique.

The reason why total protein concentration is raised in CF subjects is not evident, but may reflect either a marked rise a single protein or an rise in multiple proteins.

Presumably these protein changes would be as a consequence of inflammation or CFTR dysfunction (or both). Further analysis of the different proteins contained within EBC (the EBC proteome) was therefore warranted, as more sensitive and specific markers of CF lung disease could be identified. The results of these studies are included in the proteomics chapter (Chapter 6).

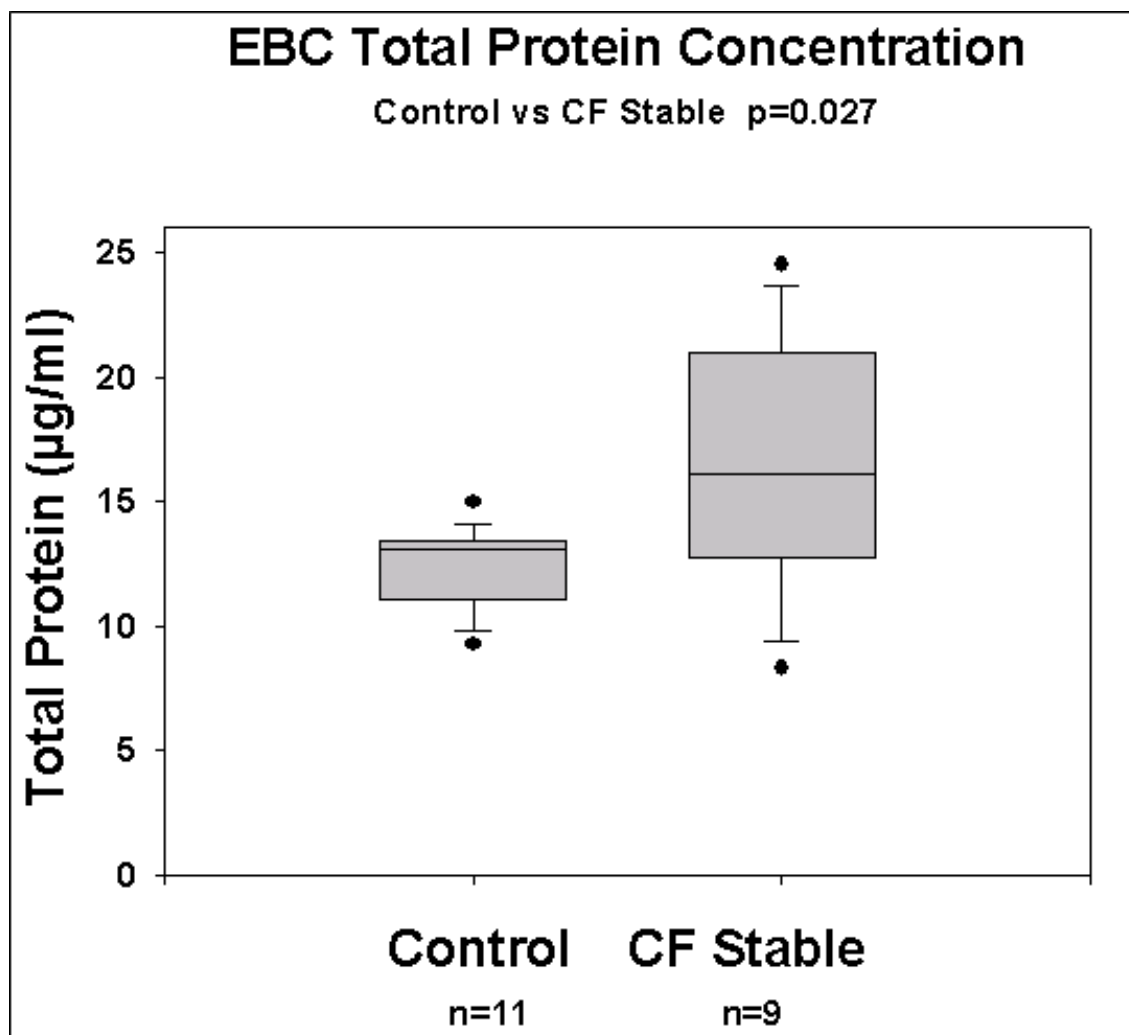


Figure 19. EBC Total Protein levels in controls and CF stable subjects.

EBC Protein levels are different between control subjects and stable CF subjects. The median value in the CF subjects was higher than any of the control subjects.

3.5 Chemical Assays - Discussion

The exhaled gases did not prove to have the ability to discriminate between CF and Non CF subjects. It could be argued that eCO should be included in a panel of markers as it could potentially report toxicity – due to increased oxidative stress. However, the toxicity would have to be considerable to change group median data and therefore should not be used in future trials.

The two nitritative stress markers, NO and nitrite were also not different between controls and CF subjects. As CFTR function seems linked through iNOS regulation and also through glutathione transport, it may be that we should anticipate a rise in either NO or nitrite during CF gene therapy trials. Therefore either of these tests should be considered for use in future trials despite the lack supporting data in this thesis.

Total protein was found to be raised in the EBC of CF subjects. This interesting finding may reflect proinflammatory proteins in the CF airway or indeed direct markers of CFTR function. These data led the direction of this thesis towards further assay of proteins. A dual approach was used, using previously published cytokines and chemokines as well as taking an unbiased proteomic approach to identify further biomarkers of CF lung disease.

4.0 – EBC - Enzyme Immunoassay Techniques

4.0 – EBC - Enzyme Immunoassay Techniques

4.0.1 Enzyme Linked Immunosorbant Assay Techniques

4.1 Proteins

4.1.1 Interleukin 8

Interleukin 8 is a potent cytokine responsible for neutrophil recruitment into the lung. As it is a proinflammatory chemokine, it may be a sensitive marker of the neutrophil influx of CF lung disease. To this end, it would be of importance to assay interleukin 8 using non-invasive techniques. IL-8 is produced in the lung predominately by macrophages, but also by epithelial cells, fibroblasts and stimulated monocytes.

Bronchoalveolar lavage studies have focussed on interleukin 8 revealing that BAL IL8 levels were 100 fold higher in CF infants when compared to non CF infants (Armstrong et al., 1995). Moreover, IL8 has been shown to be sensitive enough to detect an improvement after adenoassociated viral (aaV) gene therapy in BAL as levels fell 30 days after delivery to humans. This efficacy was lost after 30 days however, perhaps reflecting aaV neutralisation by antibodies (Moss et al., 2004). It has previously been detected in EBC from subjects with CF (33% of subjects) using standard ELISA techniques (Cunningham et al., 2000), therefore efforts were made to improve sensitivity of detection.

4.1.2 Methods

As interleukin 8 release is an important proinflammatory pathway leading to neutrophil recruitment, a major component of the work of this thesis was on validating an assay for it. Interleukins 6, 10, 1 β and TNF α were the other cytokines identified for investigation. For all five cytokines, an ultrasensitive Enzyme Linked Immunosorbant Assay (ELISA) was used. These assays allow detection of very low quantities of compounds. For IL 8, the ultrasensitive ELISA from Biosource (Biosource International, USA) is sensitive to 0.39pg/ml in serum and cell culture supernatants. Using this assay, combined with 10 fold concentration by freeze drying techniques, it is theoretically possible to detect IL 8 at the femtogram level (39 fg/ml).

4.1.2.1 Biosource ultrasensitive ELISA – Interleukins 8, 6, 10, 1 β and TNF α ,

A kit based sandwich assay was used. The antibody (e.g. to Human Interleukin 8) was coated to a microtitre plate. A standard curve (in ultrapure water) using recombinant standard was generated (IL 8 - 0.39pg/ml to 25pg/ml). Samples were added to wells on the same microtitre plate (100µl of sample). The plate was then incubated at 37°C for 2 hours to bind antibody to antigen. Next, the plate was washed 4 times and a secondary biotinylated antibody added and incubated for 1 hour. The plate was again washed 4 times. Next streptavidin-horseradish peroxidase was bound to the biotinylated antibody for 30 minutes and washed 4 times. This complex was detected by the addition of chromogen to generate a blue colour (15-45 minutes). After stopping the reaction with hydrochloric acid, a yellow colour was generated, and the plate read in a spectrophotometer at 450nm. Data were extrapolated using sigma stat to perform linear regression from the standard curve.

4.1.2.1.1 Standard Curve diluents

The recommended diluent for serum and cell line assays was the Biosource diluent buffer. The contents of this diluent are not in the public domain but are likely to contain serum and detergents. Often cell line and serum samples require dilution to be in the middle of the detection range of the ELISA, and are diluted using the recommended diluent. EBC has a low protein concentration, cannot be diluted, and therefore the standard curve used for ELISAs was validated to ensure that appropriate extrapolation of data was performed.

The data published on EBC interleukins have protein levels at (or below) the limit of detection of most ELISAs. The methods with regard to standard curves were not stated in these publications (Bucchioni et al., 2003, Carpagnano et al., 2002a, Shahid et al., 2002).

4.1.2.1.2 Methods

Pooled EBC from healthy volunteers collected using the Jaeger Ecoscreen was used for validation of the standard curve. Biosource diluent and pooled EBC were compared by spiking with different concentrations of recombinant interleukin 6 and 8 (500, 250, 125, 62.5, 31.2, 15.6 and 7.8 pg/ml) to yield a standard curve. See figure 20 and 21.

4.1.2.1.3 Results

The figures show higher OD readings for any given quantity of interleukin 6 and 8 when using Biosource diluent rather than EBC. This means that when Biosource buffer is used to generate a standard curve, any extrapolated protein concentrations will be falsely low as EBC appears to have a negative effect on IL8 detection. The effect is less marked with interleukin 6. See Figures 20 and 21.

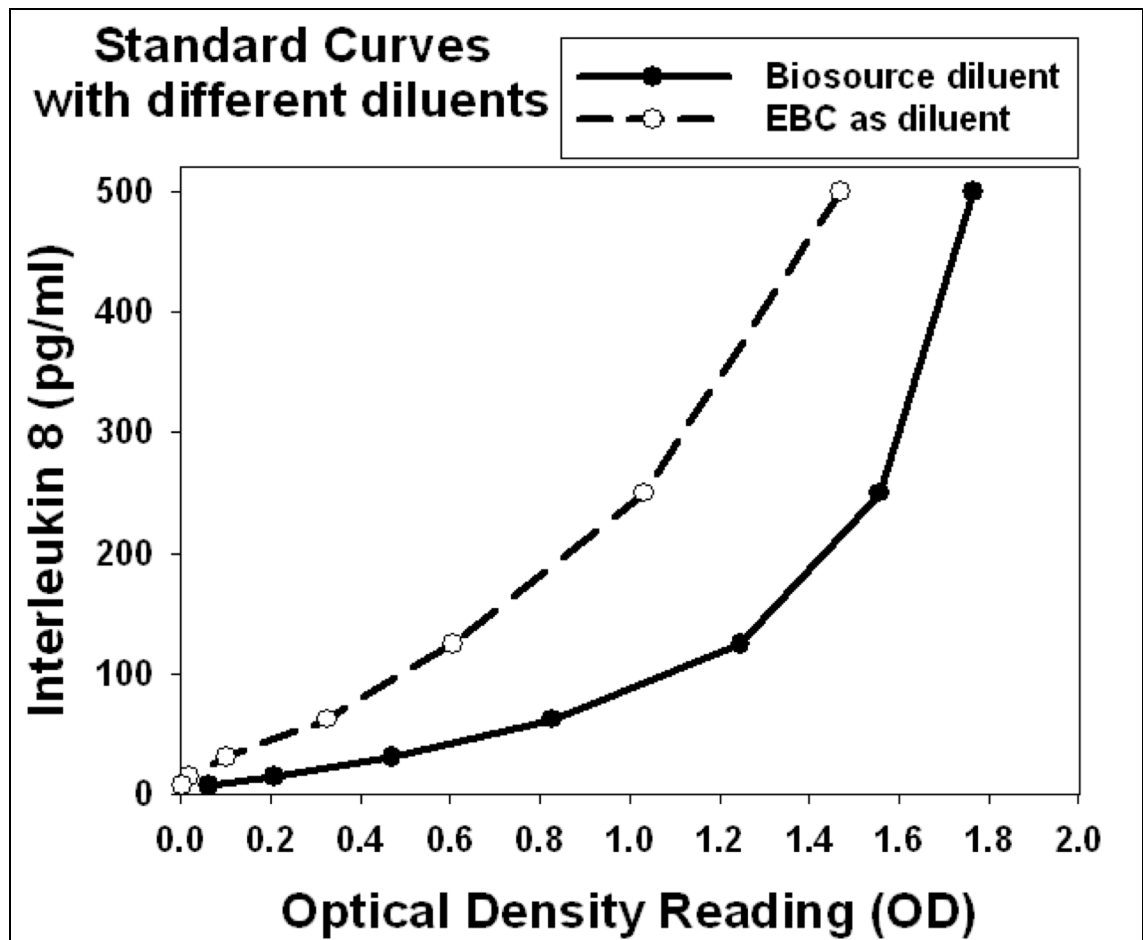


Figure 20. Interleukin 8 Standard curve with EBC and Biosource buffer as diluents.

Lower OD readings were generated when EBC was used instead of biosource buffer, meaning that when EBC is used, samples compared to a standard curve in biosource buffer would give false high results.

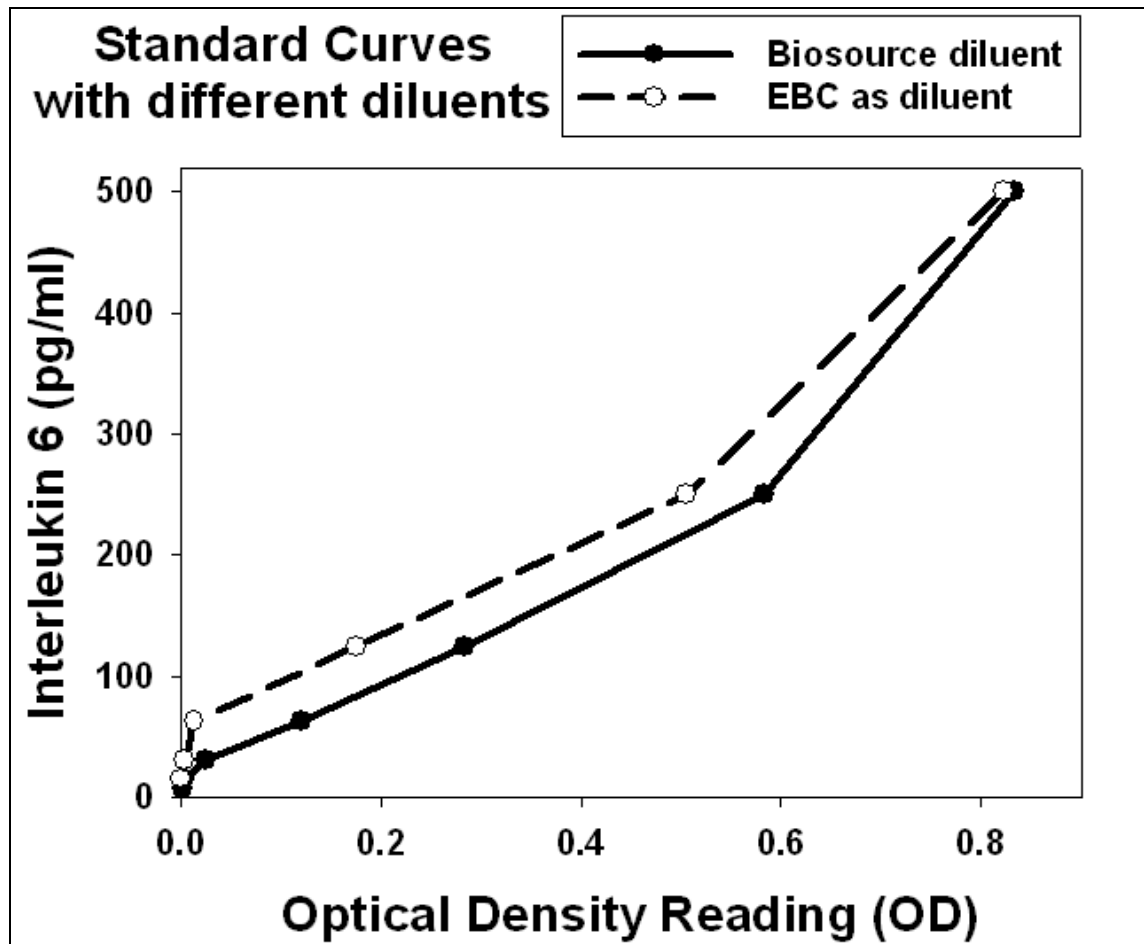


Figure 21. Interleukin 6 Standard curve with EBC and Biosource diluent.

The difference between OD reading using EBC and Biosource buffer were not as great, meaning that results compared to Biosource buffer standard curve would be less inaccurate than with interleukin 8.

4.1.2.1.4 Discussion

These data are important as they imply that using the recommended assay diluent will give false low levels of interleukin 6 and 8 in EBC. The reason for this is that linear regression analysis will be performed on incorrect OD readings. One reason the OD readings may be higher in the Biosource diluent group is that it is likely to contain protein and therefore there will be more non-specific binding. This would not be a problem for serum and cell lines as these are diluted in assay diluents and are at higher points on the curve, however EBC is not diluted as it would then be below the level of detection of the assay.

The difference in OD readings was not so great with IL6, and therefore the importance of using an appropriate standard curve would seem to be less significant with IL6. Presumably the difference is due to the binding characteristics of the ELISA epitopes selected for the two assays, and differences in non-specific binding of these two epitopes.

Errors in extrapolation from an inappropriate standard curve does not explain why some groups detected in interleukins in EBC (see 4.1.2.3.3 for explanation). and why these data have not been repeatable in our hands. It also does not explain the significant difference between diseased and non diseased groups that other groups have found e.g. interleukin 4 and 6 raised in disease groups compared to controls (Bucchioni et al., 2003, Carpagnano et al., 2002a, Shahid et al., 2002). It is not clear therefore whether these data reflect a real difference in cytokine levels or if they reflect another difference e.g. non specific binding of other proteins.

4.1.2.3 Attempts to identify an appropriate buffer.

In an attempt to find a more suitable diluent compared to EBC, interleukin 8 was reconstituted into different buffers. The different buffers may have different advantages as listed.

The buffers tested were

- Ultrapure H₂O – Theoretically the most similar to EBC as EBC is reported to have very low ion concentrations.
- 0.9% Na Cl – Physiological level of saline solution. The theoretical advantage of isotonic saline would be that protein folding of cytokines would be physiological, therefore potentially exposing the binding site which the ELISA monoclonal antibody was raised against.
- PBS – may have the advantage of being appropriate buffer to allow protein refolding to expose appropriate epitope (in the same way as 0.9% Na Cl).
- Biosource Buffer – Serum based buffer supplied with Interleukin 8 kit. Appearance would be in keeping with serum based buffer with added detergents, formulation not in public domain.
- KPL Buffer – Commercially available buffer, formulation not in public domain. This buffer was clear rather than pink (biosource buffer) and therefore its colour would not have any effect on OD readings
- EBC – Pooled EBC collected from healthy subjects for comparison

4.1.2.3.1 Methods

The above listed substances were compared as buffers by spiking with different concentrations of recombinant interleukin 8 (250, 125, 62.5, 31.2, 15.6, 7.8 and 3.75 pg/ml) to create a standard curve.

4.1.2.3.2 Results

The buffers all gave different OD reading for any given quantity of interleukin 8. Biosource buffer was most similar to EBC, but all buffers used gave higher OD readings than EBC. (Figure 22)

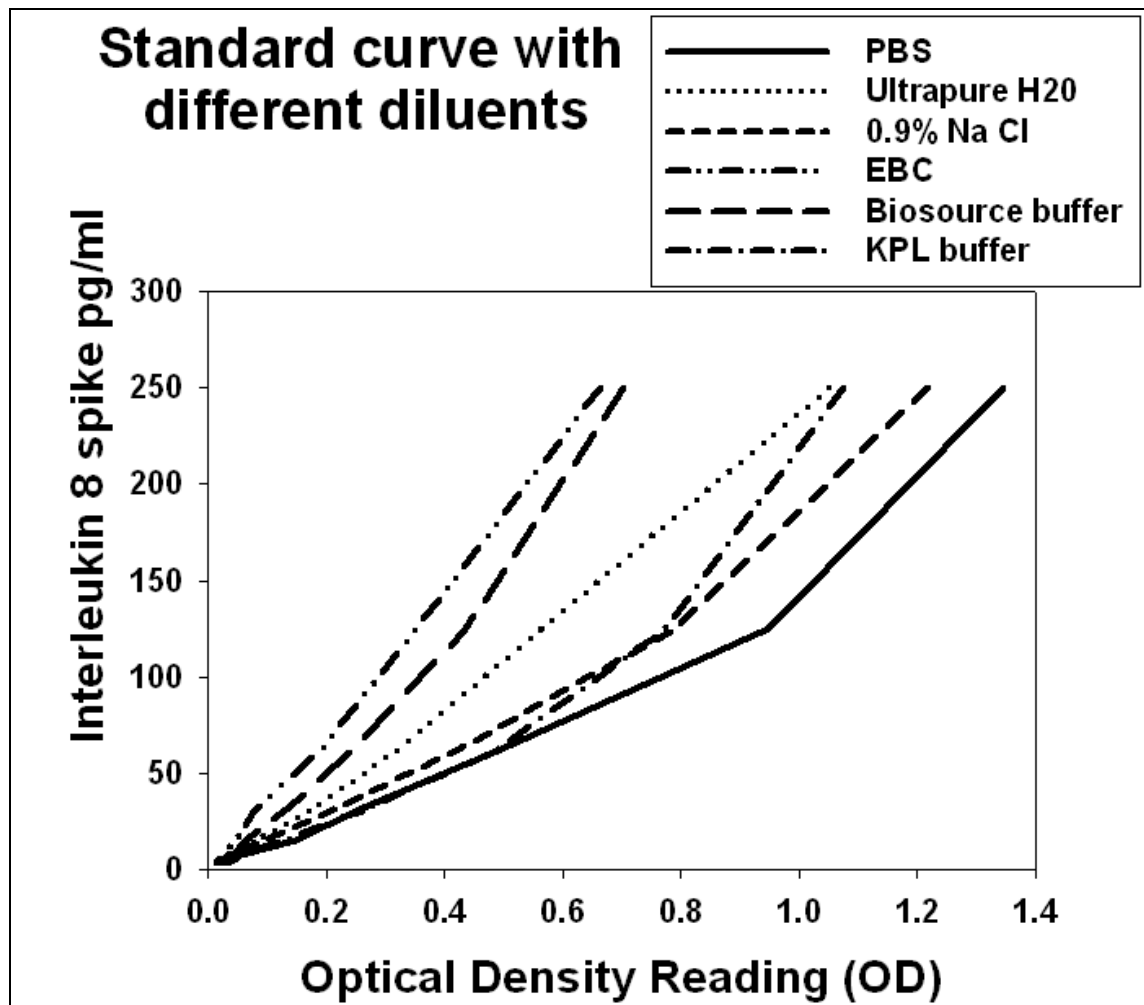


Figure 22. Interleukin 8 with different diluents.

EBC is seen to give the lowest OD reading for any given concentration of interleukin 8.

4.1.2.3.3 Discussion

These data show that assays of spiked interleukin 8 are less sensitive using EBC as the diluent than in any other buffer tested. As the concentrations of cytokines in EBC are low, it is necessary to add larger volumes of EBC to an ELISA than necessary for more concentrated biological fluids e.g. serum or BAL. EBC appears to have an inhibitory effect on ELISA detection as this experiment shows, and therefore it is vital that an appropriate standard curve is used.

The explanation for EBC giving lower OD readings than other diluents could be due to interleukin 8 degradation by EBC, IL8 epitopes for ELISA antibodies being masked by substances in EBC or that compounds in EBC interfere with the ELISA technique. As multiple washing steps are in place after allowing 2 hours of initial binding of interleukin 8 in the sample to the well, it seems more likely that degradation of IL8 or inhibition of the binding process are the more relevant factors rather than non-specific binding (which should remove non-IL8 proteins).

Importantly the difference in OD reading between EBC, biosource buffer and ultrapure water could potentially explain why other groups found detectable levels of interleukins which were not repeatable in our hands. If the standard curve is generated in biosource buffer and EBC is lyophilised and resuspended in ultrapure water, any extrapolation from the standard curve will give a false high level of interleukin in the samples. As lyophilisation and resuspension in water was the technique applied in other studies, this would be a potential explanation for other groups results (Edme et al., 2008, Bayley et al., 2008, Bodini et al., 2007, Sack et al., 2006, Carpagnano et al., 2003, Carpagnano et al., 2002b). In addition this shift in OD reading would explain why all data generated in these studies are at the lower limit of detection of ELISAs.

4.1.3 Compounds to optimise ELISA signal

These results suggest a detrimental of EBC effects on IL8 detection. The postulated mechanisms were degradation of IL8, inhibition of binding of IL8 or non specific binding to IL8. The first two were addressed by addition of chemicals listed below and the later by adding protein in the form of bovine serum albumin.

4.1.3.1 Compounds to prevent IL8 degradation.

A number of different protease inhibitors and phosphatase inhibitors were added to EBC in an attempt to avoid degradation of Interleukins. Previous work has shown that this strategy works for induced sputum, increasing the detection of interleukin 5 by at least 60 fold using an ELISA (Kelly et al., 2001). In cystic fibrosis sputum, there is an imbalance between protease and antiprotease activity, with a particular excess of neutrophil elastase and proteinase 3 (Witko-Sarsat et al., 1999). Therefore, it is conceivable that there is unopposed protease activity in the EBC of CF subjects.

The following Protease inhibitors were tested – AEBSF, Aprotinin, Leupeptin, Bestatin, Pepstatin A, E- 64 and EDTA.

The following Phosphatase Inhibitors were tested - Microcystin LR, Cantharidin, P-bromotetramisole, Sodium Vanadate, Sodium Molybdate, Sodium Tartrate and Imidazole.

As EBC has a very low protein and bicarbonate concentration, it has fewer biological buffers than serum or plasma (most ELISA kits are designed for Serum/Plasma). Two biological buffers were used in an attempt to improve IL8 detection.

The buffers tested were Bovine Serum Albumin and HEPES.

4.1.3.1.1 Compounds to enhance binding by improving epitope exposure

The sputum produced in CF has a relatively high quantity of DNA (Henke et al., 2007), this can bind to interleukin 8 and therefore may mask the epitope for binding to the antibody and microtitre plate. Previous studies in sputum have shown that adding DNase can enhance the IL8 signal (Perks and Shute, 2000, Shah et al., 1996).

Additionally, actin may bind and mask the IL8 epitope. Gelsolin can be used to cleave actin and lead to increased epitope exposure (Perks and Shute, 2000).

The enzymes tested were DNase 1 and Gelsolin.

4.1.3.1.2 Methods

The above listed substances were compared by adding to standard curves with different concentrations of recombinant interleukin 8 (250, 125, 62.5, 31.2, 15.6, 7.8 and 3.75 pg/ml). The two buffers compared were EBC and ultrapure water to clarify whether there was any effect on EBC. Pooled EBC collected from healthy subjects was used to generate the EBC standard curve.

4.1.3.1.3 Results of studies to optimise interleukin 8 assay

Aprotinin and BSA caused increased OD readings. PBS, EDTA and HEPES caused reduction in OD readings. These changes were the same in both EBC and ultrapure water. No other compounds listed above altered OD readings.

4.1.3.1.4 Discussion

No compound had a differential effect on OD readings in EBC. As there was no difference between water and EBC, this implies that there is no benefit in using these chemical agents to improve detection i.e. any effects are on the assay rather than an effect on EBC. No method caused a reduction in IL8 degradation or improved binding of IL8.

In support of non-specific binding to the ELISA well, the addition of the proteins aprotinin and BSA will give higher OD readings, an effect similar to that seen using Biosource buffer.

4.1.4 Freeze Drying - with spiked samples

When directly assaying patient samples for IL8 in EBC, all results are below the level of detection of the assay. Freeze-drying allows concentration of the sample from 1ml to 100µl – i.e. a 10-fold increase in concentration.

4.1.4.1 Methods

To assess detection of interleukin 8 after freeze drying and after concentration, spiking experiments were performed. Pooled EBC was collected from healthy volunteers. Recombinant interleukin 8 was added to freshly collected EBC to a concentration of

20pg/ml. This was then split into 3 groups. 1 aliquot was frozen and assayed 24 hours later (frozen), 1 aliquot freeze dried and reconstituted in its original volume using ultrapure water and assayed 24 hours later (FD-original volume), and the third aliquot was freeze dried, reconstituted in 1/10th of its original volume using ultrapure water (10 fold concentration) and assayed 24 hours later (FD-1/10th volume).

4.1.4.2 Results

From the 20pg/ml fresh spike, in the sample frozen and assayed 24 hours later, the median return was 0.789pg/ml (3 replicates, range 0.719 – 1.09). In the samples which were freeze dried and then reconstituted back to their original volume the median return was 1.399pg/ml (10 replicates, range 1.384-1.431). The highest return was with freeze drying and reconstitution in 1/10th of original volume and the return was 3.49pg/ml (10 replicates, range 3.38-3.49). The maximum return from the 20pg/ml spike was less than 3.82 pg/ml, which is only 19% of the original quantity of IL8 added to the EBC (figure 23).

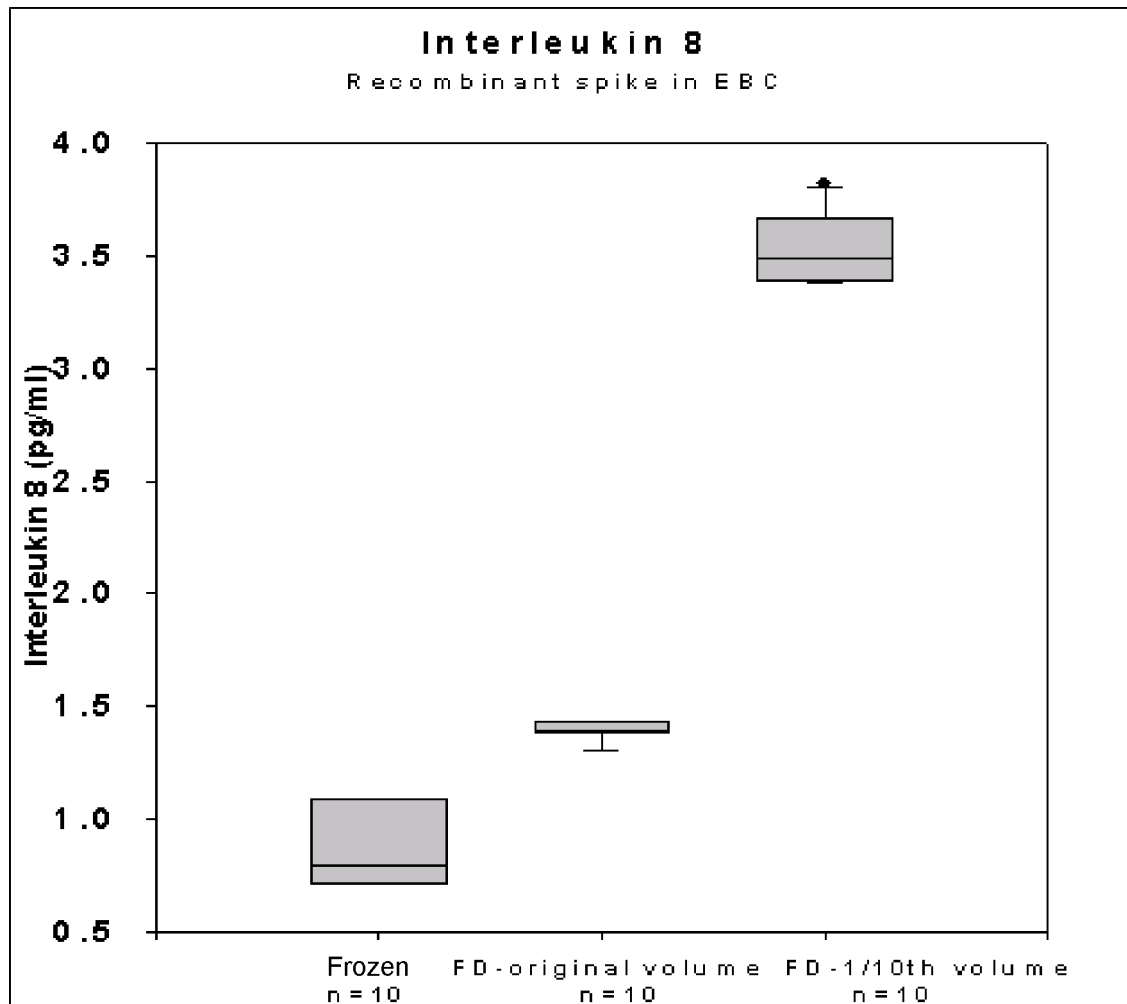


Figure 23. 20 pg spike of interleukin 8 in EBC, fresh sample, freeze dried sample and freeze dried and concentrated 10 fold sample. Data are expressed in pg/ml.

Pooled EBC was collected and divided into 1ml aliquots. 20pg of recombinant interleukin 8 was added to each sample. Then the samples were processed in three different ways:-

1. Sample frozen immediately and thawed 24 hours later and then assayed (frozen).
2. Sample freeze-dried immediately and then stored until assay 24 hours later then reconstituted in 1ml of ultrapure water i.e. to original volume (“FD 1X recons” in figure).
3. Sample freeze-dried immediately and then stored until assay 24 hours later then reconstituted in 100µl of ultrapure water i.e. 1/10th of original volume (“FD and 10X conc” in figure).

4.1.4.3 Discussion

The highest return on the original 20pg of recombinant interleukin 8 was less than 20% when using the freeze drying and concentrating method. This technique did show good reproducibility between samples (n=10), however return was poor. Therefore, recombinant interleukin 8 does not appear to be stable in EBC despite freeze drying techniques, although some improvement in detection was made. Methods to try to reduce interleukin 8 degradation, unmask IL8 epitope and remove interfering compounds were unsuccessful.

As no optimal method giving full return was identified, the method chosen for EBC collection of clinical samples was freeze-drying and 10 fold concentration. The choice of diluent after lyophilisation is a subject for debate. As other groups had used reconstitution in ultrapure water with success, we chose to do the same (Edme et al., 2008, Bayley et al., 2008, Bodini et al., 2007, Sack et al., 2006, Carpagnano et al., 2003, Carpagnano et al., 2002b). However after interpreting the previous experiments, we chose to also make up the standard curve in ultrapure water to ensure appropriate extrapolation of data.

4.2 EBC IL 8 in CF vs Control Adults.

Introduction.

Despite best efforts, an optimal assay to measure IL8 levels in EBC was not identified. As an IL8 assay would be beneficial, the method which gave the best results (i.e. highest return on previous experiment) was chosen for testing with CF and control EBC.

Methods

The technique used was to snap freeze 1ml of EBC immediately after collection, then freeze dry and concentrate then sample in 1/10 of original volume (by reconstituting in 100µl of ultra pure water), and assay using a Biosource ultrasensitive ELISA, with the standard curve made up in ultrapure water. Ultrapure water was used as diluent and standard curve to ensure appropriate extrapolation of end-data.

Subjects.

The subjects included in this study were 8 healthy control subjects (adults) and 7 stable CF adults and 10 CF adults during infective exacerbation. 1 ml of EBC was collected from each subject for this assay.

4.2.1 Results

Interleukin 8 was detectable in 36% of samples at very low levels (all less than 3pg/ml) and was not detectable i.e. below the level of detection of the assay in the other 64% of samples. See Figure 24.

There was no difference in interleukin 8 levels between control and CF subjects. We found no difference in interleukin 8 between stable CF adults (n=7, Median 0.00 (IQR 0.00-1.08)) and control subjects (n=8, Median 0.15 (IQR 0.00-0.59)), p=0.694. There was no difference CF adults during exacerbation (n=10, Median 0.00 (IQR 0.00-0.67)) and control subjects (n=8, Median 0.15 (IQR 0.00-0.59)), p=0.688.

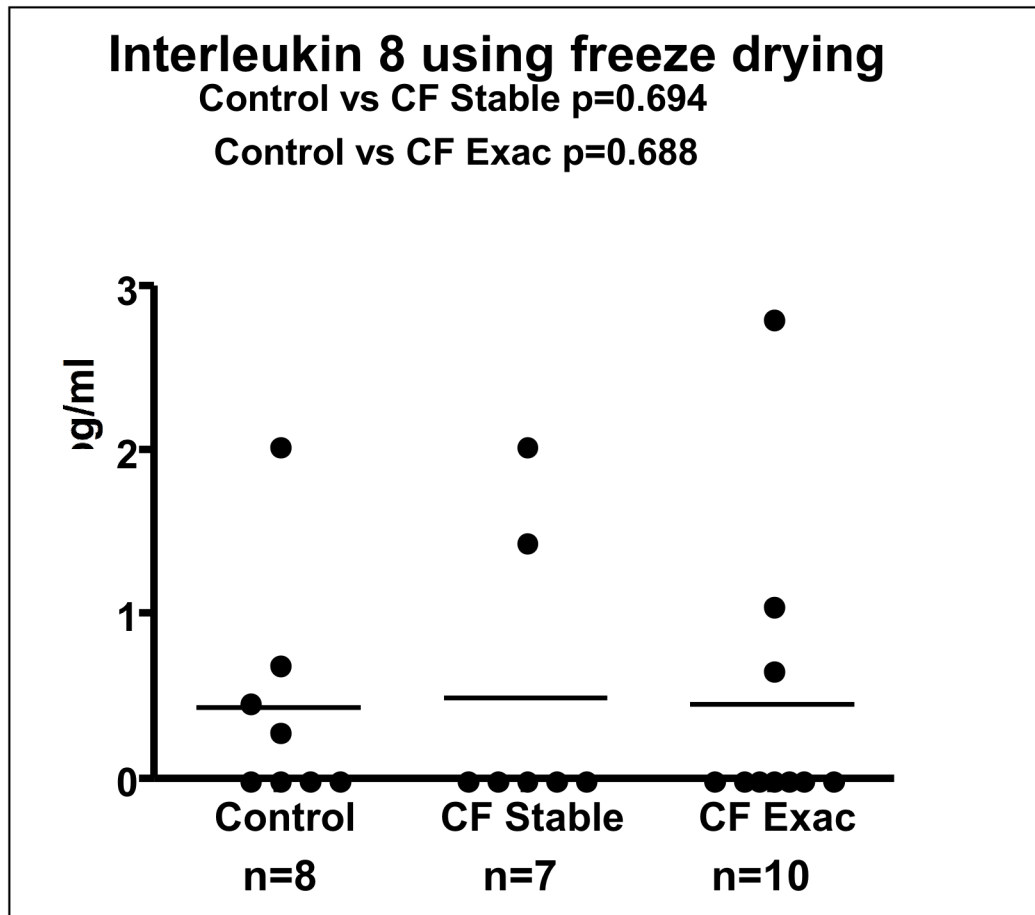


Figure 24. EBC IL8 levels in control vs CF stable vs CF exacerbation data after freeze drying and concentrating 10 fold.

As the majority of the CF samples were below the limit of detection of the assay (64%), individual data are plotted. There were no differences in the data from control and CF subjects. The limit of detection of the assay was 0.39pg/ml.

4.2.1 Discussion

These data are in agreement with a previous paediatric study, which found interleukin 8 to be detectable in one third of samples (Cunningham et al., 2000). By using an ultrasensitive ELISA (10 fold more sensitive) and concentrating sample 10 fold, our assay should have been more sensitive.

As the positive samples were all at the lower end of detection by ELISA, a more sensitive detection technique was needed to ensure that the positive results were genuine. Despite best efforts it was not possible to stabilise interleukin 8 in EBC, and it was only detectable in just over one third of samples. Therefore these analysis techniques did not prove to be robust enough to be recommended for use in future gene therapy trial. More sensitive analysis techniques were sought, and the chosen route was to use proteomics techniques for further analysis of EBC.

4.3 Other Cytokines

4.3.1 Interleukins 6

Interleukin 6 has both pro and anti-inflammatory effects. It is known to be raised in CF BAL (Bonfield et al., 1995). Interleukin 6 may be useful in monitoring toxicity of CF gene therapy in that it has previously been raised after dosing with viral gene therapy vectors and predicted clinical deterioration (Ben-Gary et al., 2002, Reix et al., 2002).

4.3.2 Interleukin 10

Interleukin 10 is an anti-inflammatory cytokine, and it has been shown to be reduced in mouse and human CF BAL (Bonfield et al., 1995). It has been postulated that interleukin 10 polymorphisms correlate with different disease phenotypes, are important in defence against bacterial and fungal colonisation (Casaulta et al., 2003, Virella-Lowell et al., 2004) and are an inhibitors of inteleukin 8 secretion (Tabary et al., 2003).

4.3.3 Interleukin 1 beta and Tumour Necrosis Factor alpha (TNF α)

These cytokines are released after exposure to bacterial exotoxins and endotoxins and are therefore relevant to CF lung disease where there is chronic colonisation by bacteria (Staugas et al., 1992). Their relevance is that they form part of the proinflammatory profile of cystic fibrosis lung disease and have been shown in vitro to cause a reduction in reporter gene (Bastonero et al., 2005).

4.3.4 Interferon gamma (INF γ)

This cytokine is secreted in response to stimulation with bacterial LPS, and this response has been shown to be reduced in CF cell lines and to have variable results in CF nasal biopsies (Moss et al., 2000, Wojnarowski et al., 1999).

4.3.5 Methods

Ultrasensitive ELISAs from Biosource were used and 100ul of EBC from 14 healthy controls and 10 subjects with Cystic Fibrosis were used for each assay.

4.3.6 Results for cytokines - IL6, IL10, IL1-beta, INF gamma and TNF alpha

All CF (n=10) and control (n=14) EBC samples had results below the limit of detection of the assay.

4.4 Discussion about ELISA

No cytokines were reproducibly detectable in EBC. After concentration using freeze-drying techniques, interleukin 8 was sometimes detectable. However, even using this technique only 36% of samples had detectable levels of interleukin 8. Assays of interleukin 6, interleukin 10, interleukin 1-beta, interferon gamma and tumour necrosis factor alpha were unsuccessful. More sensitive and specific analytical tools were needed to assay proteins in EBC. Accordingly attempts to analyse proteins were made using more sensitive techniques. A proteomics approach was made using mass spectrometry. See chapter 6.

4.5 Competitive Enzyme Immunoassay (EIAs)

4.5.1 Lipid mediators

4.5.1 Introduction

Leukotrienes are synthesised from arachidonic acid. LTB₄ is a potent mediator. It causes chemotaxis and chemokinesis in human polymorphonuclear leukocytes at subnanomolar levels. At higher concentrations, it leads to neutrophil aggregation and degranulation as well as superoxide anion production. LTB₄ is present and rises after withdrawal of steroids in asthma patients in exhaled breath condensate. LTE₄ is generated predominately by mast cells and eosinophils and causes mucus secretion and airway smooth muscle contraction. LTE₄ levels in CF spontaneous sputum correlate (Leff, 2000) with disease severity (FEV₁) (Montuschi et al., 2000). It is most predominant leukotriene present in CF sputum (75%) whereas LTB₄ is predominant in asthmatic sputum (65%) (Montuschi and Barnes, 2002b).

4.5.2 Competitive Enzyme Immunoassay- LTB₄ and LTE₄

These assays are similar to ELISA in that binding to antibody is required. Unlike ELISA they use a competitive technique whereby the analyte competes with a tracer chemical for binding to assay well. This technique is used for assay of leukotrienes B₄ / E₄, 8-isoprostane and nitrotyrosine.

This technique was used to assay LTB₄ and LTE₄. A kit based enzyme immunoassay (EIA) was used (Cayman Chemicals, USA). The technique is the same for each assay, and will be described for LTB₄. The LTB₄ assay is a competitive assay based on the competition between LTB₄ and an LTB₄-acetylcholinesterase conjugate (LTB₄ tracer) for a limited amount of LTB₄ antiserum. Because the concentration of LTB₄ varies, the amount of LTB₄ tracer that is able to bind to the LTB₄ antiserum will be inversely proportional to the concentration of LTB₄ in the well. This antibody-LTB₄ complex binds to a mouse monoclonal anti-rabbit antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to anticholinesterase) is added to the well. The product of this enzymatic reaction has a yellow colour and can be read on a microplate reader at 420 nm (actual peak 412nm). The intensity of OD reading is proportional to

the amount of LTB4 tracer bound to the well, and this is inversely proportional to the amount of free LTB4 present in the well during the incubation. Linear regression analysis is used for data extrapolation, and negative correlations between Optical Density (OD) readings and concentration of standards.

As the assay used was a commercially available kit using competitive binding techniques, attempts to optimize the standard curve (as with interleukin 8) were not possible.

Subjects

The subjects included in this study were 6 healthy controls where fresh EBC was collected immediately before assay (all within 1 hour). The remainder of the sample was stored for 1 week at -80°C and the assay was then repeated.

EBC samples were also collected over a 3 month period from 20 healthy control subjects and 20 CF subjects. These samples were stored at minus 80°C until being assayed.

4.5.3 Results

Leukotrienes were detectable in fresh EBC median 27.4 pg/ml (n=6, IQR 15.4-39.1). When assayed after storage of 1 week in the minus 80°C freezer, LTB4 was not detectable in any of the samples.

When analysis stored samples, 3 of the 40 samples showed LTB4 at the lower limit of detection of the assay, and the remaining 37 were below limit of detection of the assay. Therefore statistical analysis was not performed due to failure of detection.

4.5.4 Discussion

Although LTB4 was detectable in fresh breath of healthy control subjects, it was not detectable after being stored for 1 week. This means that LTB4 was not stable and would have required immediate assay. This would have been prohibitively expensive both in assay costs (one assay kit per day) and in staff time to perform the assay. Therefore, it was not possible to run large numbers of fresh samples, and no further investigation of LTB4 was performed.

4.6 8-Isoprostane assay

8 - Isoprostane is a member of the eicosanoid family. It is produced by oxidation of tissue phospholipids by oxygen radicals. It is a potent pulmonary vasoconstrictor and has been implicated as a causative mediator in pulmonary oxygen toxicity. 8-isoprostane has been proposed as a marker of oxidative stress and has been shown to be raised in the EBC of Cystic Fibrosis and asthmatic subjects (Montuschi et al., 2000, Zanconato et al., 2004). As a marker of oxidative stress, it has several advantages in that it is chemically stable, is formed *in vivo* at sites of free radical activity, and is specific for lipid peroxidation.

4.6.1 Methods

An EIA supplied by Cayman Chemicals (Ann Arbor, USA) was used. This was a competitive assay where 8-isoprostane in the sample competed with an 8-isoprostane tracer compound. Butylated Hydroxytoluene was added to the samples to prevent *ex vivo* oxidation after the sample was collected.

Test samples of EBC collected from 6 healthy volunteers were used to optimise the assay. The results were at the lower limit of detection, and therefore freeze-drying was used to concentrate the samples ten fold.

Samples were collected from 9 control subjects and 18 adults with cystic fibrosis when they were deemed clinically stable. Samples were also assayed from 8 subjects who were in-patients with an infective exacerbation of cystic fibrosis.

4.6.2 Results

We found no difference in 8-Isoprostane levels between stable CF adults (n=18, Median 2.65 (IQR 1.80-4.20)) and control subjects (n=9, Median 3.13 (IQR 2.38-8.52)), p=0.258. There was no difference CF adults during exacerbation (n=8, Median 2.75 (IQR 2.02-3.75)) and control subjects (n=9, Median 3.13 (IQR 2.38-8.52)), p=0.386 or CF stable subjects p=0.978. See figure 25.

4.6.3 Discussion

There were no differences between the study groups. Numerically the 8-isoprostane levels were lower in the CF groups, which is contrary to the hypothesis that it can be

used to report on the increased oxidative state of the CF lung. The assay used was a competitive assay and therefore EBC could be having a detrimental effect on binding of the reporter chemical, giving a false positive signal.

Another technique would be to employ gas chromatography mass spectrometry as a more sensitive (and potentially more specific) technique than the EIA used here. GC MS techniques were not available, and the data from this study would suggest that further research using EIA techniques to assay isoprostanes in EBC are not warranted in the context of Cystic Fibrosis gene therapy trials.

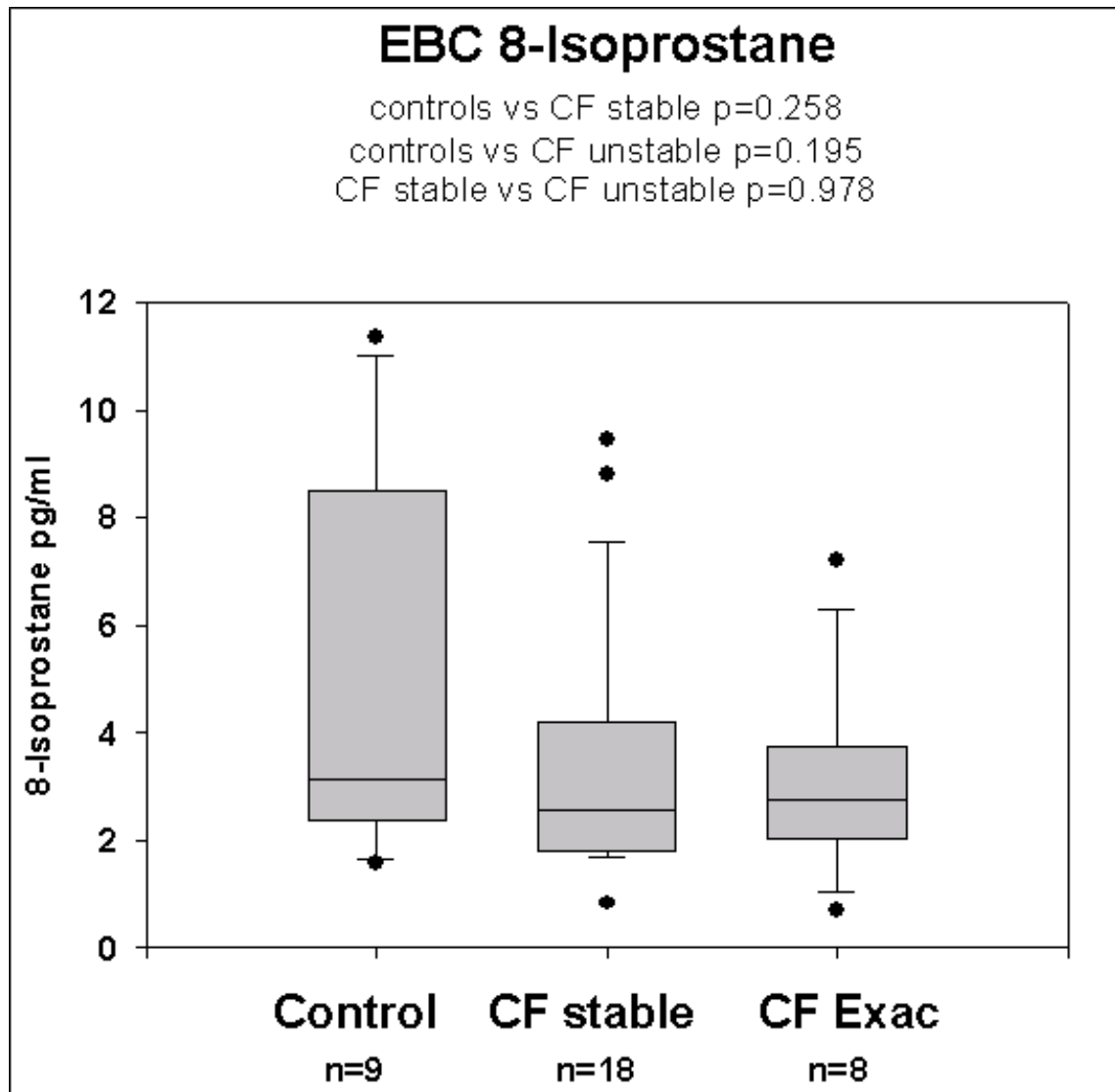


Figure 25. EBC 8 Isoprostane levels in control subjects vs CF stable subjects vs CF exacerbation subjects.

The levels of 8-isoprostane were not different between the groups.

4.7 Nitrotyrosine

Nitrotyrosine is a stable end product of the reaction between the amino acid tyrosine and reactive nitrogen species. It could therefore be used as a stable end product assay of nitrative stress. It has been investigated in EBC and has previously found to be raised in stable steroid naive asthmatics (Hanazawa et al., 2000), after cigarette smoking (Balint et al., 2001a) and in Cystic Fibrosis (Balint et al., 2001b). In the studies in this thesis the markers of nitrative stress, eNO and Nitrite have not been raised, despite results of previous studies. It was hoped that the marker nitrotyrosine would reflect the increased nitrative stress in the CF lung and therefore was studied in our CF population.

4.7.1 Methods

An EIA supplied by Cayman Chemicals was used. The technique employed was again a competitive assay where nitrotyrosine in the sample competed with labelled nitrotyrosine for a set quantity of antibody.

Samples were collected from 13 control subjects and 9 adults with cystic fibrosis when they were deemed clinically stable. Samples were also assayed from 8 subjects who were in-patients with an infective exacerbation of cystic fibrosis.

4.7.2 Results

There was no difference in EBC nitrotyrosine levels between stable CF adults (n=9, Median 4.46 ng/ml (IQR 1.84-6.23)) and control subjects (n=13, Median 4.16 ng/ml (IQR 1.84-6.23)), p=1.000. There was no difference CF adults during exacerbation (n=8, Median 3.93 ng/ml (IQR 2.05-7.39)) and control subjects (n=13, Median 4.16 ng/ml (IQR 1.84-6.23)), p=0.800 or with CF stable patients. See Figure 26.

4.7.3 Discussion

No difference in nitrotyrosine was found between any of the groups and therefore this assay will not be of use in future gene therapy trials. As this was a competitive assay, the concerns about EBC having a negative effect on the binding of antibody to antigen in the well apply i.e the levels of nitrotyrosine reported may be a false positive result.

It is interesting to note that all three markers of nitrate stress eNO, EBC nitrite and EBC nitrotyrosine cannot differentiate between subjects with cystic fibrosis and healthy control subjects. This raises the possibility that either there is no difference between controls and cystic fibrosis subjects (true negative result) or that the available techniques were unable to detect a difference (false negative studies). There is a need for more sensitive assays to clarify the answer to this question.

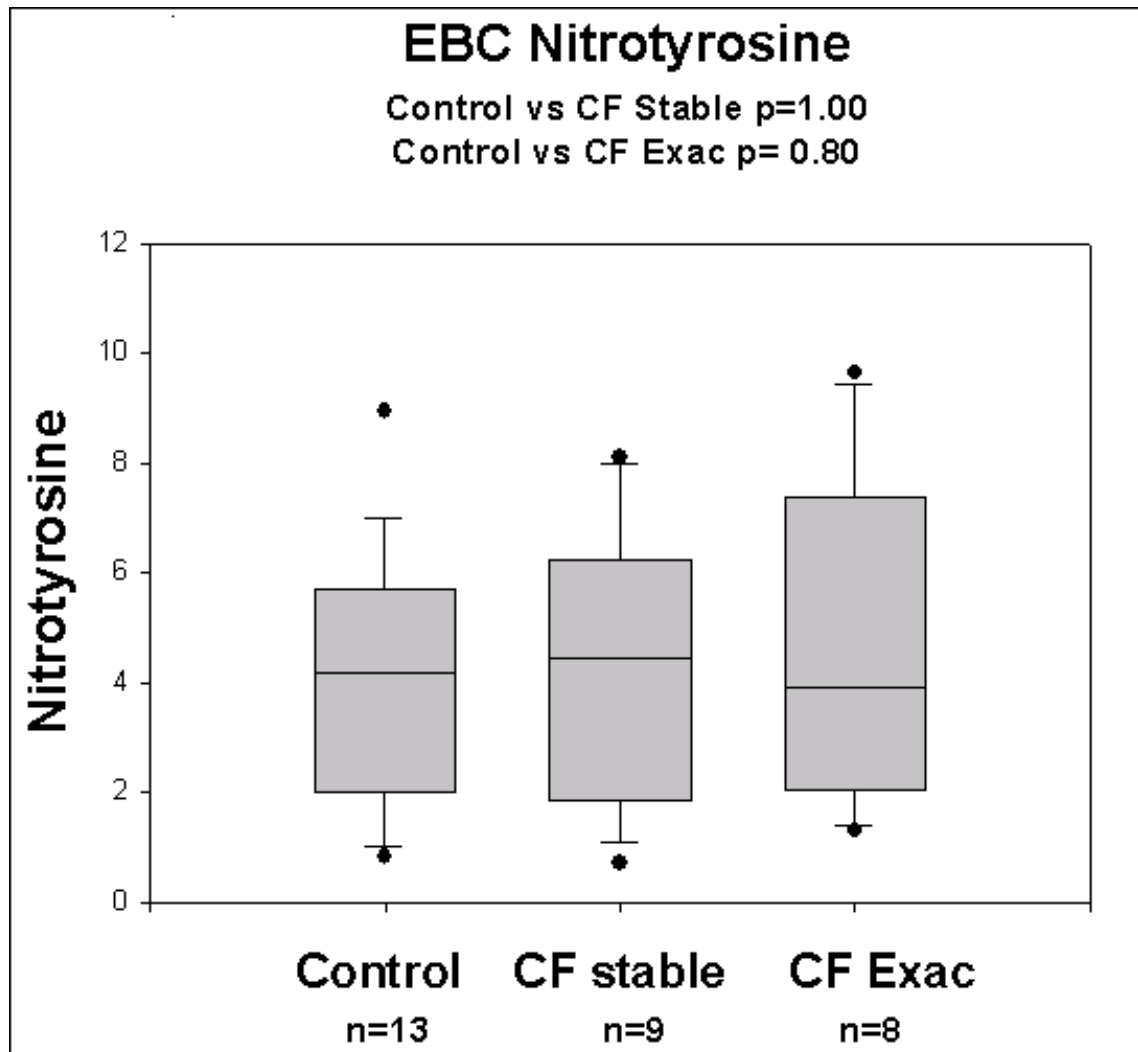


Figure 26. EBC Nitrotyrosine levels in controls, CF stable subjects CF Exacerbation subjects.

There were no differences in EBC nitrotyrosine levels in patients with CF compared to controls.

4.8 Enzyme immunoassay techniques – ELISA and EIA

4.8.1 Discussion

The use of ELISA and EIA techniques to discriminate between CF and controls subjects was unsuccessful. Despite a significant effort to try to optimise ELISA for assay of cytokines in breath, in particular interleukin 8, it was not successful. Concerns around degradation of sample, compounds masking epitopes or substances interfering with binding of sample to antibodies remain. The most significant concern is that any signal detected was always at the lower end of detection of ELISA and therefore confidence about detection was not possible. At best interleukin 8 was only detected in 36% of samples and did not differentiate between CF and control groups. Other cytokines were not detectable.

EIA proved more successful in detection, with nitrotyrosine and 8 isoprostane detected in all subjects. However the results did not discriminate a cystic fibrosis subject from a control subject. Also the results were positive at low levels. Concern about the competitive nature of the assay raised questions about the genuine presence of these compounds in EBC. There seemed to be a rather narrow spread of data in all subjects, and it might be concluded that the results were false positives.

The positive results reported other groups could not be repeated. The main issue with other studies were the possible use of inappropriate standard curves for ELISAs, leading to extrapolation that compounds were present at levels higher than reality (i.e. false positive results). Interpretation of data in previous studies appeared potentially flawed in that if no compound was detected, a fictitious level was given to those samples. Despite these concerns, there does not appear to be an explanation as to why researchers found differences between disease groups, and therefore it would be appropriate to conclude that their studies were measuring real differences but the techniques have proven to be unrepeatable.

As there were no differences detected between CF and control subjects, these assays should not be used for EBC analysis in future gene therapy trials. More sensitive and specific analytical tools are needed to assay compounds in EBC. In view of the raised total protein in EBC, proteomic techniques were investigated (chapter 6).

5.0 EBC - Ion Measurements

5.0 EBC - Ion Measurements

5.1 pH

5.1.1 Introduction

The pH of exhaled breath condensate was found to be low in asthmatic subjects when compared to controls (Hunt et al., 2000). This is taken as reflecting severity of airways inflammation, since the pH normalizes with treatment of the exacerbation. The reduction in pH may be attributed to an increase either in an as yet unidentified inflammatory acid, or by diminished buffering capacity of EBC.

An increase in acidity of biological fluids in response to a proinflammatory stimulus is recognised to occur (in humans) In chronic suppuration such as in bacterial infection of the pleural cavity (empyema), a lowering of fluid pH is seen (Chavalittamrong et al., 1979). The lowering of pH in empyema is thought to be due to leukocyte phagocytosis and bacterial metabolism (Sahn et al., 1983).

In cystic fibrosis there is chronic suppuration with bacterial colonisation and neutrophil inflammation, and through these mechanisms the ASL pH may be lower than that of controls. In addition, as CFTR has been proposed as a bicarbonate channel, the ASL in CF may be further acidified due to reduced levels of bicarbonate (Ballard et al., 1999). It was, therefore, hypothesised that the pH of EBC in cystic fibrosis subjects may be lower than healthy controls.

5.1.2 Methods

Patients were recruited at the Paediatric and Adult Cystic Fibrosis services in Edinburgh. Control subjects were recruited from staff at the Adult Centre and children attending the fracture clinic in the Paediatric Centre.

Two types of pH meter were used in this work. Initially a glass electrode was used (Orion pH probe, USA) with a pH millivolt meter. This was capable of measuring the pH in 650µl of EBC accurate to 0.01pH. As this is a large volume of EBC, a solid-state electrode system was tested. This required 50µl of EBC (pH Boy, Camlab, UK) which was sensitive to 0.1 pH point (figure 27). Both systems required two-point calibration before each use and required a stable temperature of sample before analysis as

temperature affects conductivity, therefore samples were allowed to warm to room temperature.

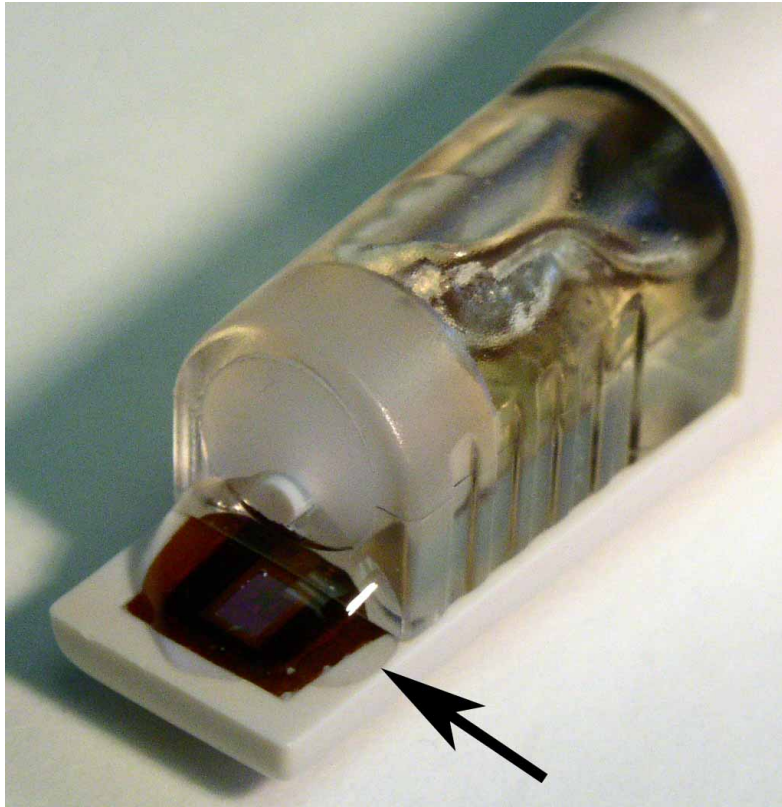


Figure 27. pH Boy ion selective electrode.

50 μ l of EBC is sufficient to cover both electrodes of the pH boy. This technique was sensitive to 0.1 pH point. The arrow shows a meniscus of EBC covering the electrodes.

5.1.2.1 Deaeration of samples

A difference in methodologies between this CF study and previously published asthma studies was performed (Hunt et al., 2000). Hunt chose to deaerate the samples and assay them later after freezing. The purpose of this was to remove dissolved CO₂ (bicarbonate). In CF studies, the importance of assaying bicarbonate is that it is secreted by CFTR and therefore may report on CFTR function. In this thesis, the samples were assayed immediately (without deaeration) in the hope that bicarbonate would be held in solution by surface tension and therefore would contribute to EBC pH.

To test whether deaeration affected pH of EBC, 6 samples were tested - three from healthy controls and three from subjects with stable cystic fibrosis attending clinic. Although deaeration changed the numerical value of pH, the order of results between all subjects was identical and there was a strong correlation between pH before and after deaeration ($r=0.67$, $p=0.000048$).

5.1.2.2 pH Boy vs Standard pH meter

The two pH meters were compared with EBC from 6 subjects - three from healthy controls and three from subjects with stable cystic fibrosis attending clinic. A strong correlation was noted ($r=0.999$, $p<0.0001$, $n=6$). Therefore, the pH boy was chosen as the preferred method of analysis as 50 μ l of sample was required as compared to 650 μ l (standard pH meter).

5.1.3 Results

EBC pH

Adults data.

EBC pH was lower in stable CF adults when compared to their age matched control group: (CF Stable – n=26, Median 5.84, IQR 5.48-6.16 vs Control – n=14, Median 6.1, IQR 5.9-6.21) p=0.047. (Table 4) (figure 28)

EBC collected at time of exacerbation had a lower pH in adults with CF when compared to controls: (CF Exacerbation – n=14, Median 5.37, IQR 4.96-5.66 vs. Control – n=14, Median 6.1, IQR 5.9-6.21) p=<0.001. (Table 4) (figure 28)

EBC pH was not lower during infective exacerbations compared to the stable CF patients in adults: (CF Exacerbation – n=14, Median 5.37, IQR 4.96-5.66) vs. (CF Stable – n=26, Median 5.84, IQR 5.48-6.16) p=0.005. (Table 4)(Figure 28)

Children's data.

EBC pH was not lower in stable CF children when compared to their age matched control group: (CF Stable – n=12, Median 5.9, IQR 5.50-6.15 vs. Control – n=20, Median 5.9, IQR 5.8-6.25) p=0.546. (Table 5) (figure 29)

EBC collected at time of exacerbation had a lower pH in children with CF when compared to controls: (CF Exacerbation – n=8, Median 5.35, IQR 5.15-5.75) vs. (Control – n=20, Median 5.9, IQR 5.8-6.25) p=0.007. (Table 5) (figure 29)

EBC pH was not lower during infective exacerbations compared to the stable CF patients in children: (CF Exacerbation – n=8, Median 5.35, IQR 5.15-5.75) vs. (CF Stable – n=12, Median 5.9, IQR 5.50-6.15) p=0.028. (Table 5)(Figure 29)

Combined adults and children's data.

Combined adult and childrens data EBC pH was not lower in stable CF patients (n=38, 5.90 median, IQR 5.48-6.16, p=0.064) compared to controls (n=34, 6.03 median, IQR 5.80-6.21). (Table 6)

Combined adult and childrens data EBC pH was lower in those with an exacerbation (n=22, 5.37 median, IQR 5.00-5.70 p=<0.001) compared to controls (n=34, 6.03 median, IQR 5.80-6.21). (Table 6)

In each subject group (controls, CF stable, CF exacerbation), there was no difference in EBC pH when adults were compared to children (Control p=0.401, CF Stable p=0.950 and CF Exacerbation p=0.394) although the data for each childrens group had wider interquartile ranges than the adults.

Subject	n=	Median	Interquartile range (IQR)
Controls	14	6.1	5.90-6.21
CF patients (Stable)	26	5.84	5.48-6.16
CF Patients (Exacerbation)	14	5.37	4.96-5.66

Table 4 – EBC pH – Adults data

Subject	n=	Median	Interquartile range (IQR)
Controls	20	5.9	5.80-6.25
CF patients (Stable)	12	5.9	5.50-6.15
CF Patients (Exacerbation)	8	5.35	5.15-5.75

Table 5 – EBC pH – Children’s data

Subject	n=	Median	Interquartile range (IQR)
Controls	34	6.03	5.80-6.21
CF patients (Stable)	38	5.90	5.60-6.20
CF Patients (Exacerbation)	22	5.37	5.00-5.70

Table 6 – EBC pH – Combined data

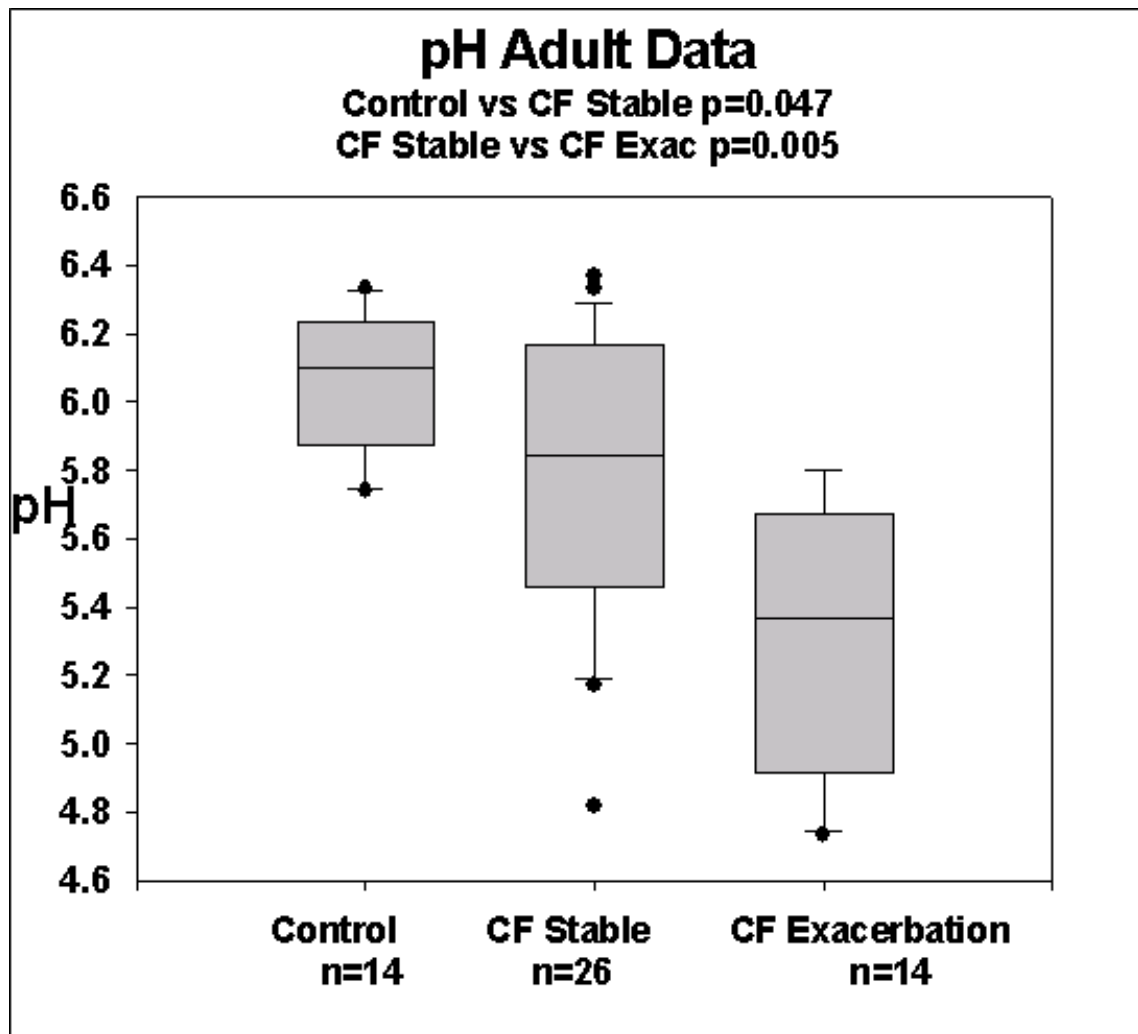


Figure 28. Adults - EBC pH levels in controls, CF stable subjects and CF subjects with exacerbation.

EBC pH was lower in both stable CF subjects ($p=0.047$) and CF subjects with exacerbation ($p<0.001$) and CF subjects with exacerbation had lower EBC pH than stable CF subjects ($p=0.005$).

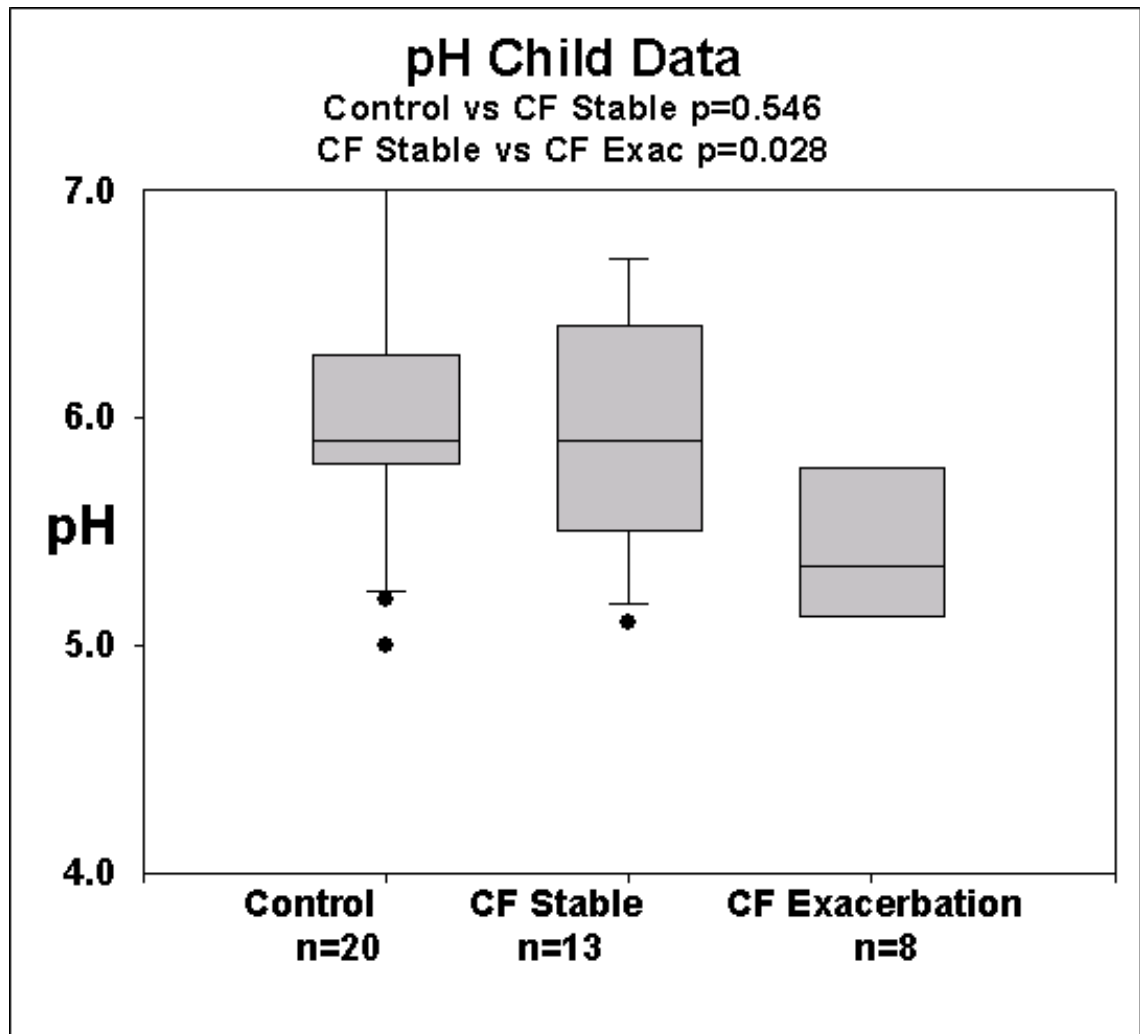


Figure 29. Children - EBC pH levels in controls, CF stable subjects and CF subjects with exacerbation.

EBC pH was not lower in stable CF subjects ($p=0.546$), but was lower in CF subjects with exacerbation ($p=0.007$)

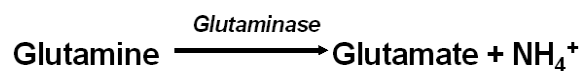
5.1.4 Discussion

Discussion of these results can be found in combination with EBC ammonium data after the EBC ammonium section.

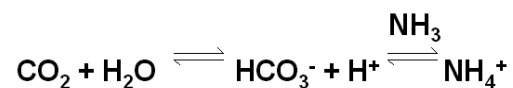
5.2 Ammonium

5.2.1 Introduction

Ammonium is one of the major biological buffers (the others being bicarbonate and proteins). Levels of Ammonium have been found to be reduced in the EBC of asthmatic subjects (Hunt et al., 2002). Lowering of Ammonium has been attributed to inhibition of the acid/base homeostatic enzyme Glutaminase. Glutaminase converts glutamine to glutamate with the formation of ammonium. Its expression can be induced in respiratory epithelial cells by an acid environment (Hunt et al., 2002). Ammonium is a weak buffer with a pKa of 9.0, and therefore when generated will neutralise any free acid. However, glutaminase expression, and thereby ammonium production, can be inhibited by proinflammatory cytokines including TNF alpha and Interferon gamma. Therefore, in the CF airway these cytokines could affect ammonium production (Gaston et al., 2002).



Another further possible source of Ammonium in the CF airway is production by denitrifying bacteria such as *Pseudomonas aeruginosa* (*pseudomonas* generates ammonia gas from nitrites), generating ammonium ions via reaction with CO₂ and H₂O (Gaston et al., 2002).



5.2.2 Methods

5.2.2.1 Ion selective electrodes

Ion selective electrodes allow the assay of different ions at very low levels. Their general application is to assay purity of drinking water, and they can assay down to 0.01 parts per million.

An ammonium electrode was used in this work to quantify ammonium in EBC. Sodium, Chloride, Potassium were also assayed using this technique and will be discussed later in the chapter. The Ammonium electrode (Jenway, UK) was attached to a sensitive millivolt meter (Jenway 3345 Ion meter, UK). As the volume of EBC was limited, a technique of inverting the electrode was used (figure 30). Then 130 μ l of EBC was applied to the surface (being held on by surface tension) (See figure 30).

The electrode functions by allowing the selective diffusion of the appropriate ion through a semi permeable membrane. The conductivity through this membrane was measured using a millivoltmeter. Because the relevant ion diffuses through the membrane to create the voltage, the voltage generated reflected diffusion kinetics, i.e. a linear change in voltage would reflect an exponential change in ion concentration.

A standard curve for extrapolation was made using serial dilution of ammonium standard. With every 10 fold dilution of ammonium standard, the voltage dropped in a linear fashion. When the voltage change became non-linear the limit of sensitivity was reached. For ammonium the voltage became non-linear at 0.01ppm and so the lower limit of detection was 0.1ppm (figure 31).

As with measurements of pH, temperature affects conductivity (and diffusion). As diffusion occurs from the sample, and solute concentrations are low in EBC, some drifting of voltage was seen. This may be due to progressive loss of ions through the membrane, altering the concentration. To avoid artefact due to this, the voltmeter recording was always taken at 10 seconds.

Calibration using solutions of ions at 1000, 100, 10, 1 and 0.1 parts per million were used as the standards.

5.2.1.2 Coefficient of variation

If many samples were assayed at once, a new standard curve was generated every 20 samples. To calculate coefficient of variation of Ammonium assay, 100 standards were assayed at 10 (550 μ M) and 100 ppm (5500 μ M). Coefficient of variation for this analysis technique was 0.7% and 1.3% respectively.

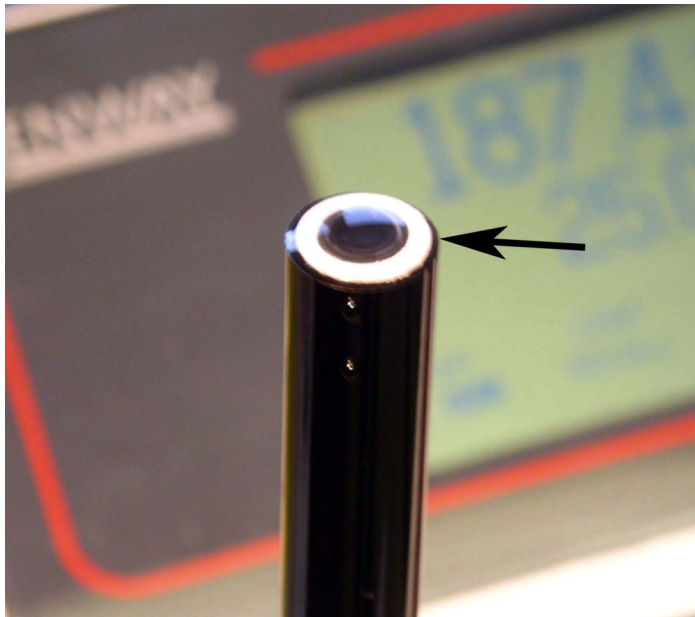


Figure 30. Jenway Ion selective electrode.

This image shows the quantitation of ammonium in 120 μ l of EBC. The electrode was inverted (turned upside down) and then fluid was applied to the tip (arrow shows EBC onto of tip) and this was held in place by surface tension.

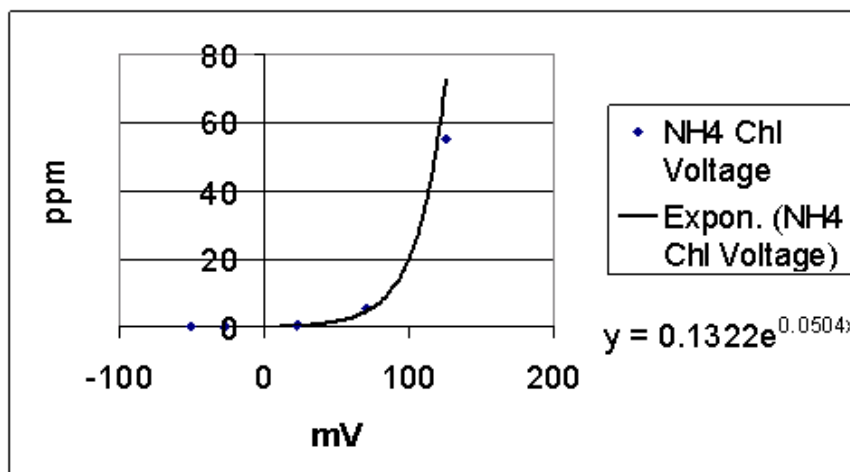


Figure 31. Generation of standard curve for regression analysis (Ammonium Chloride)

As ion diffusion across a membrane obeys nernstian kinetics, regression analysis of the standard curve to moles is non-linear (exponential).

5.2.3 Results

EBC Ammonium

Adults data.

EBC Ammonium was lower in stable CF adults when compared to their age matched control group: (CF Stable – n=10, Median 160, IQR 40-230 vs Control – n=18, Median 536, IQR 380-1030) p=0.002. (Table 7)(Figure 32)

EBC collected at time of exacerbation had a lower Ammonium in adults with CF when compared to controls: (CF Exacerbation – n=14, Median 70, IQR 56-200 vs. Control – n=18, Median 536, IQR 380-1030) p=<0.001. (Table 7)(Figure 32)

Ammonium was not lower during infective exacerbations compared to the stable CF patients in adults: (CF Exacerbation – n=14, Median 70, IQR 56-200) vs. CF Stable – n=10, Median 160, IQR 40-230) p=0.519. (Table 7)(Figure 32)

Children's data.

EBC Ammonium was lower in stable CF children when compared to their age matched control group: (CF Stable – n=12, Median 230, IQR 205-520 vs. Control – n=44, Median 428, IQR 276-638) p=0.040. (Table 8)(Figure 33)

EBC collected at time of exacerbation had a lower Ammonium in children with CF when compared to controls (CF Exacerbation – n=7, Median 290, IQR 180-415) vs. (Control – n=44, Median 428, IQR 276-637) p=0.070. (Table 8)(Figure 33)

Ammonium was not lower during infective exacerbations compared to the stable CF patients in children: (CF Exacerbation – n=7, Median 290, IQR 180-415 vs. CF Stable – n=12, Median 230, IQR 205-520) p=0.583. (Table 8)(Figure 33)

Combined data.

Combined adult and childrens data EBC Ammonium was lower in both stable CF subjects (n=22, 220µM median, IQR 150-330µM, p=<0.001) and those with an

exacerbation (n=20, 140 μ M median, IQR 60-350 μ M p=<0.001) compared to controls (n=62, 480 μ M median, IQR 311-730 μ M) (Table 9).

Subject	n=	Median μM	Interquartile range (IQR) μM
Controls	18	536	380-1030
CF patients (Stable)	10	160	40-230
CF Patients (Exacerbation)	14	70	56-200

Table 7 – EBC ammonium - Adult data

Subject	n=	Median μM	Interquartile range (IQR) μM
Controls	62	480	311-730
CF patients (Stable)	22	220	150-330
CF Patients (Exacerbation)	20	140	60-350

Table 8 – EBC ammonium – Children’s data

Subject	n=	Median μM	Interquartile range (IQR) μM
Controls	44	428	276-637
CF patients (Stable)	12	230	205-520
CF patients (Exacerbation)	7	290	180-415

Table 9 – EBC ammonium - Combined adults and children’s data

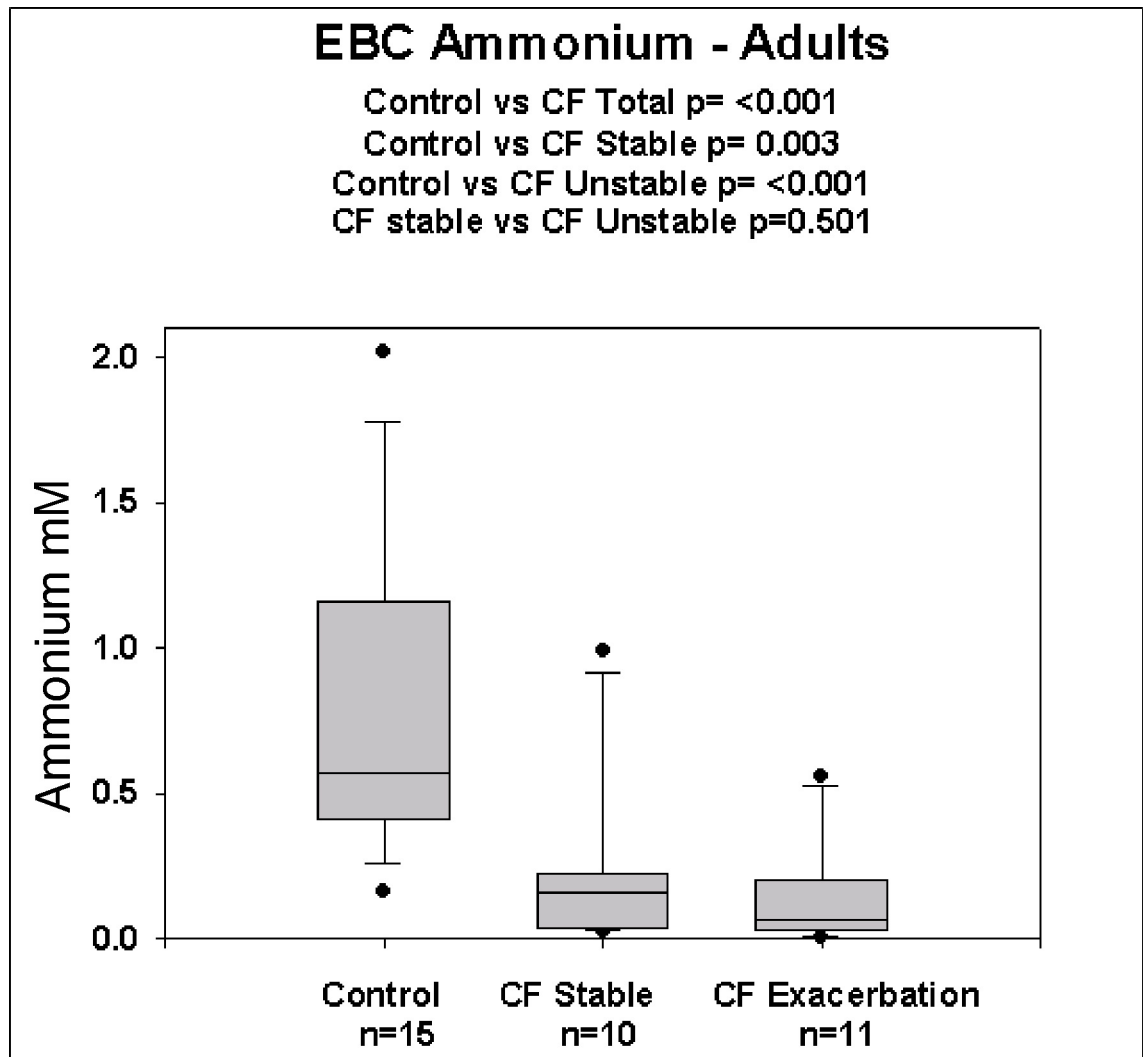


Figure 32. EBC ammonium in control and CF adults.

EBC ammonium was lower in stable CF adults ($p=0.003$) and during exacerbation ($p<0.001$) compared to their age matched control group.

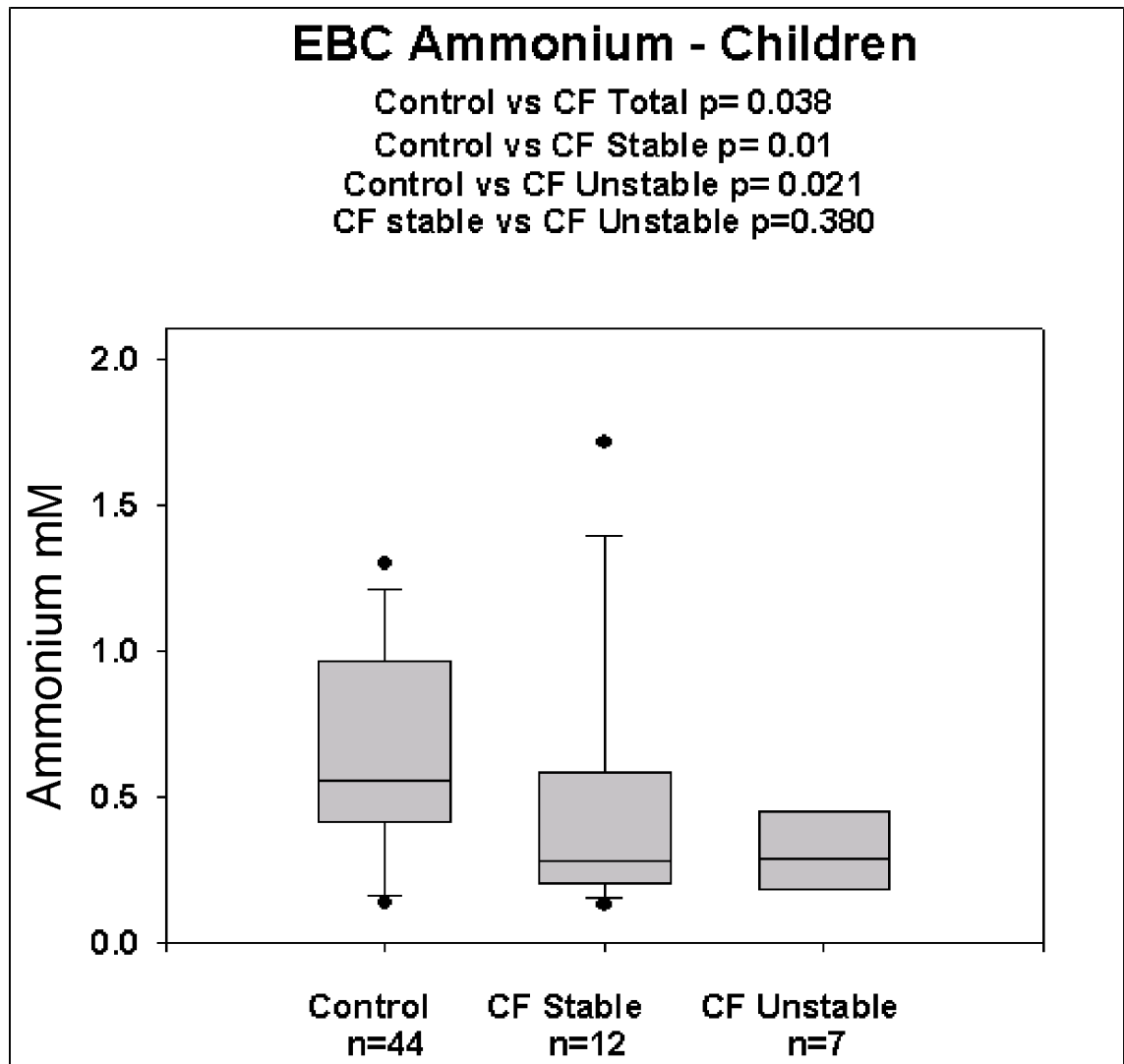


Figure 33. EBC ammonium in control and CF children.

EBC ammonium was lower in stable CF children ($p=0.040$) and during exacerbation ($p=0.070$) when compared to controls.

5.3 Discussion – pH and ammonium data combined.

This study shows that pH of EBC in stable adults with Cystic Fibrosis is reduced compared to controls. In stable children with CF, in contrast, there was no difference in pH compared to controls. This difference between adults and children raises the possibility that the adult CF airway is more inflamed, even when clinically well, than that of CF children.

The lower pH detected during exacerbation is likely to be reflect inflammation as EBC pH of both CF adults and children was lower during exacerbation. This would be consistent with a worsening of lung inflammation causing a reduced EBC pH, as occurred in subjects with Asthma, COPD and bronchiectasis (Hunt et al., 2000, Kostikas et al., 2002).

When we looked at the buffer ammonium, in adults and children EBC ammonium was reduced, even when stable, and did not drop any further during exacerbation.

Interestingly stable CF children had a lower EBC ammonium than controls, unlike EBC pH. It is possible that this reflects a type 2 error as the number of stable CF children recruited was lower than CF adults, more data would be needed to address this question. This lower ammonium was not different between the CF stable and CF exacerbation groups. This implies that the low pH seen in all CF subjects during exacerbation may reflect an increase in an “inflammatory” acid (or reduction in other buffer) rather than a reduction in ammonium.

It could be postulated that pH and ammonium are present in equilibrium (i.e as pH falls the ammonium buffer would fall). If this is the case, the data would imply that ammonium would fall before pH (as ammonium, but not pH is low in CF children). It is likely that only after the buffering capacity of EBC is used up (e.g. with a high quantity of an “inflammatory” acid), the pH would fall. Both a low pH and ammonium would then reflect a higher levels of inflammation, potentially as seen in adults with CF.

There was no correlation between pH and Ammonium in any disease group or age group which may reflect the fact that ammonium is low in all CF subjects. Also there was no correlation with % Predicted FEV₁, bacterial colonisation, cigarette smoking or genotype.

In each subject group the level of pH was not altered by age, as there were no statistical differences between adult and child data. A wider spread of data was noted for children and the wider spread of data was seen in control and CF subjects. This wider range potentially reflected variations in sample collection, but it is possible that in the CF subjects there was a wider variation in inflammation in children. However, ammonium was lower in the adult CF population compared to the CF children, and this may reflect the greater severity of inflammation present in the stable CF adult compared to the stable CF child.

EBC Ammonium is lower in the CF population when compared to healthy controls. One possible mechanism being the action of proinflammatory cytokines on glutaminase expression. This would also partly provide an explanation for the reduced EBC pH.

Methodological differences account for the difference in pH between these data and those previously reported by previous investigators (Hunt et al., 2000, Kostikas et al., 2002). We chose not to deaerate our samples with Argon, and measured pH immediately after sample collection. When tested, a strong correlation between the two methodologies, with and without argon, was noted. The reason for the difference was as an attempt to indirectly assay bicarbonate. By rapidly measuring pH without deaeration we hoped to hold bicarbonate in suspension due to surface tension. We anticipated that EBC pH would thereby reflect CFTR function/dysfunction. The finding that EBC pH in CF stable children was not different from their controls implies that we are not assaying any differences in bicarbonate in EBC pH in CF subjects. A difference in EBC pH in CF stable adults therefore is more likely to reflect an increase in inflammation rather than any CFTR dysfunction reflected in low bicarbonate.

Interestingly, there was no relationship between EBC pH and Ammonium. This is contrary to previous data in asthma (Hunt, Erwin et al. 2002) and implies changes in the acid/base status of EBC, which are not related to ammonium. Kostikas found a difference in EBC pH in bronchiectatics colonised with pseudomonas, compared to non pseudomonas bronchiectatics (Kostikas et al., 2002). In CF subjects there was no difference in EBC pH or Ammonium with colonising organism, implying that production of Ammonium by denitrifying bacteria such as pseudomonas does not affect EBC pH or ammonium to a detectable extent in CF EBC.

A reduction in ASL pH could lead to detrimental effects on the CF airway such as increasing mucous viscosity (Bhaskar et al., 1991, Holma, 1985), decreasing ciliary beat frequency (Clary-Meinesz et al., 1998) and directly altering the integrity of the respiratory epithelium (Giddens and Fairchild, 1972). In spite of this we found no correlation with FEV₁. FEV₁ is an insensitive measure of “short term” changes in inflammatory state of the airway in CF. Instead it reflects predominantly a lifetime of lung damage and deterioration, which is multifactorial in causation.

One potential source of ammonium is the mouth. No change in either EBC pH or ammonia was detected when control subjects were asked to hyperventilate. Indeed, as CF patients have poor dentition due to the high energy diet and mineralization defect of teeth, we would expect higher levels of ammonia than controls, as production of ammonia gas by assimilating bacteria would increase. This was not the case.

It has not been established as to which components of ASL are present in EBC. We hypothesised that we are collecting the aerosolised particles of the periciliary liquid layer of ASL, rather than the more viscous mucus layer which lies on top. There are two supporting pieces of evidence for this. Firstly, nasal ASL pH is not lower in CF subjects compared to controls (McShane et al., 2003), suggesting we are not measuring the same material. It may be possible that as the nasal epithelium is not a good model of lung inflammation or that EBC pH reflects a volatile gas, but it is also possible that EBC pH reflects a subcomponent of the ASL. Topical application of pH electrode to the airway would be measuring the resistance of the top mucus layer of ASL, and not the periciliary liquid layer beneath, whereas EBC would be sampling the microdroplet components of ASL which can aerosolise. Secondly, it has previously been shown that CF sputum has higher levels of ammonium (produced by pseudomonas) than non CF sputum (from children with neurogenic respiratory failure) (Gaston and Hunt, 2002). If EBC was sampling the entire ASL, we should expect EBC ammonium levels to be high in CF (as in CF mucus where pseudomonas exists in biofilms). However if the viscous mucus layer was resistant to aerosolisation, the periciliary layer of ASL would be the major contributor to EBC. The periciliary microenvironment of airway epithelial cells is one of the last lines of defence against invading organisms and therefore it is

conceivable that it is here that the three proton pumps responsible for acidifying ASL (Na^+/H^+ exchange, H^+ ATPase and an H^+ , K^+ ATPase) exhibit their greatest effect.

In conclusion, these data would suggest that EBC pH is low in stable CF adults and is further reduced in adults and reduced in children with CF during infective exacerbations. Ammonium is reduced in all CF subjects. These endpoints have the potential to be used as surrogate markers of the inflammatory status of CF patients. In this study, the collection and analysis of EBC proved simple and safe to perform in subjects as young as 6 years of age. For future trials of new therapies in Cystic Fibrosis, the differences between pH and Ammonium in CF subjects could be utilised. As a surrogate marker of inflammation, EBC pH is most likely to be useful in detecting any deterioration from a stable baseline in both adults and children, since the pH during CF exacerbation was highly different from the stable CF EBC pH level. EBC Ammonium may be more useful when detecting any improvement from a stable CF baseline i.e. drug trials or gene therapy trials designed to improve the steady state of the patient towards that of healthy controls, since Ammonium levels were greatly reduced compared to the levels of healthy controls. Further studies of these surrogate markers of inflammation are warranted to assess their potential application in the clinical management of CF patients.

5.4 Sodium, Chloride, Potassium (and Lactate) Ions

CF is characterised by an apically localised, ATP dependant chloride channel. Therefore assay of chloride in the airway surface liquid in a non-invasive manner would be a good way to assess CFTR function. ENaC dysfunction is also associated with CFTR dysfunction and measurement of sodium may also be helpful.

In vivo analysis ASL sodium and chloride is difficult and most data are available for nasal measurements only. Using *in vivo* micro-dialysis in mice, no difference in CF nasal ASL sodium and chloride levels was found, but potassium levels were higher in CF mice (Grubb et al., 2002). In human studies using filter paper absorption to assay sodium and chloride, no difference was found between CF subjects and controls (Knowles et al., 1997). This field of research remains controversial as methodological arguments as to which techniques truly assay ASL have been made (since ASL has a mucus and periciliary liquid layer, topical and adsorptive techniques may not be assaying entire liquid).

Therefore, as EBC offers the potential to sample the ASL, albeit in dilute form, we chose to investigate Na⁺, Cl⁻ and K⁺ levels in EBC. As there is a dilution factor (by exhaled water and variations in shedding of microdroplets) the three ions and their ratios were investigated to see if there is a CF non - CF difference.

Lactate has been suggested as the fourth main ion in EBC (Effros et al., 2002) and may be raised in cystic fibrosis due to pseudomonas metabolism of pyruvate and has been postulated to be raised in the saliva of CF subjects (Bardon, 1987, Eschbach et al., 2004).

5.4.1 Methods

Ion selective electrodes for sodium, chloride and potassium were tested. The subjects were children who were either healthy controls (who had been attending fracture clinic) and children with CF attending clinic (stable).

As well as assaying the absolute values of different ions, the ratios between them were also. This was an attempt to use the different ions as internal standards to compensate for the dilution effect that EBC would have on droplets of ASL.

5.4.2 Results

There was no difference detected with any of the ions, nor of the ratio between ions.

Sodium - No difference between CF (n=19, Median 0.8µg/l (IQR 0.5-1.2)) and Non CF (n=19, Median 1.0 (IQR0.6-3.6)) p=0.307 (Table 10)Figure – 34/A

Potassium - No difference between CF (n=19, Median 0.2µg/l (IQR 0.17-0.32)) and Non CF (n=19, Median 0.3 (IQR 0.19-0.42-)) p=0.307 (Table 12) Figure – 34/B

Chloride - No difference between CF (n=19, Median 0.1µg/l (IQR0.08-0.3)) and Non CF (n=19, Median 0.2 (IQR 0.1-0.3)) p=0.161 (Table 11)Figure – 34/C

Lactate – Majority of samples below limit of detection of assay. (Table 13)Figure – 34/D

The ratio between ions was tested and no difference was found between the control and CF population. The ratios were Na/K – p=0.110, Na/Cl p=0.221, K/Cl p=0.161. There was no difference in the total ion count of CF and control EBC Na+K+CL p=0.225. See figure 35/A-D.

Subject	n	Median $\mu\text{g/l}$	Interquartile range (IQR)
Controls	19	1	0.6-3.6
CF patients	19	0.8	0.5-1.2

Table 10. EBC Sodium levels in control and CF subjects. No difference between the groups. P=0.307

Subject	n	Median $\mu\text{g/l}$	Interquartile range (IQR)
Controls	19	0.2	0.1-0.3
CF patients	19	0.1	0.08-0.3

Table 11. EBC Chloride levels in control and CF subjects. No difference between the groups. P=0.161

Subject	n	Median $\mu\text{g/l}$	Interquartile range (IQR)
Controls	19	0.3	0.19-0.42
CF patients	19	0.2	0.17-0.32

Table 12. EBC Potassium levels in control and CF subjects. No difference between the groups. P=0.307

Subject	n	Median $\mu\text{g/l}$	Interquartile range (IQR)
Controls	19	0.0	0.00-0.15
CF patients	19	0.0	0.00-0.05

Table 13. EBC Lactate levels in control and CF subjects. No difference between the groups. P=0.109

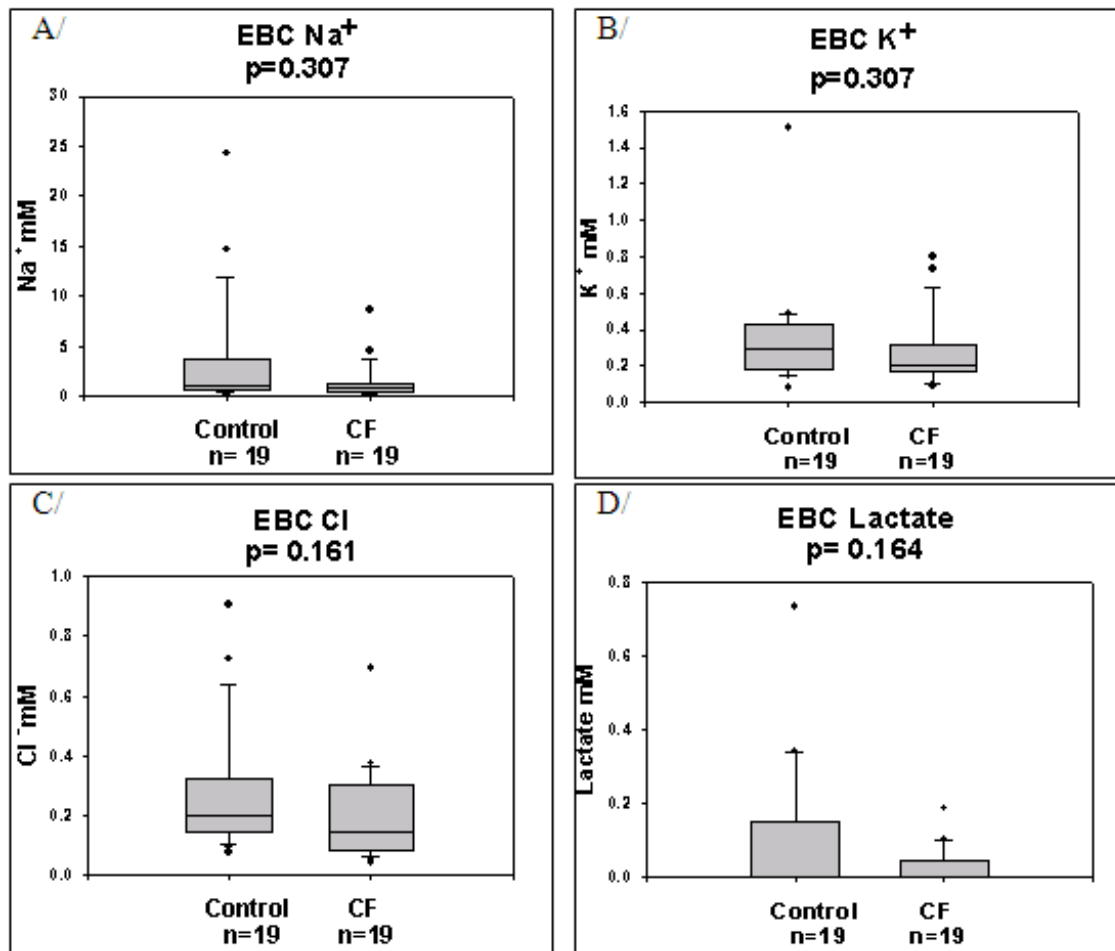


Figure 34/

- A. EBC ions Sodium - No difference between CF and controls.
- B. EBC Potassium - No difference between CF and controls.
- C. EBC Chloride- No difference between CF and controls.
- D. EBC Lactate- No difference between CF and controls. Lactate below limit of detection in majority of samples.

No difference between controls and CF for Sodium, Potassium, Chloride or lactate.

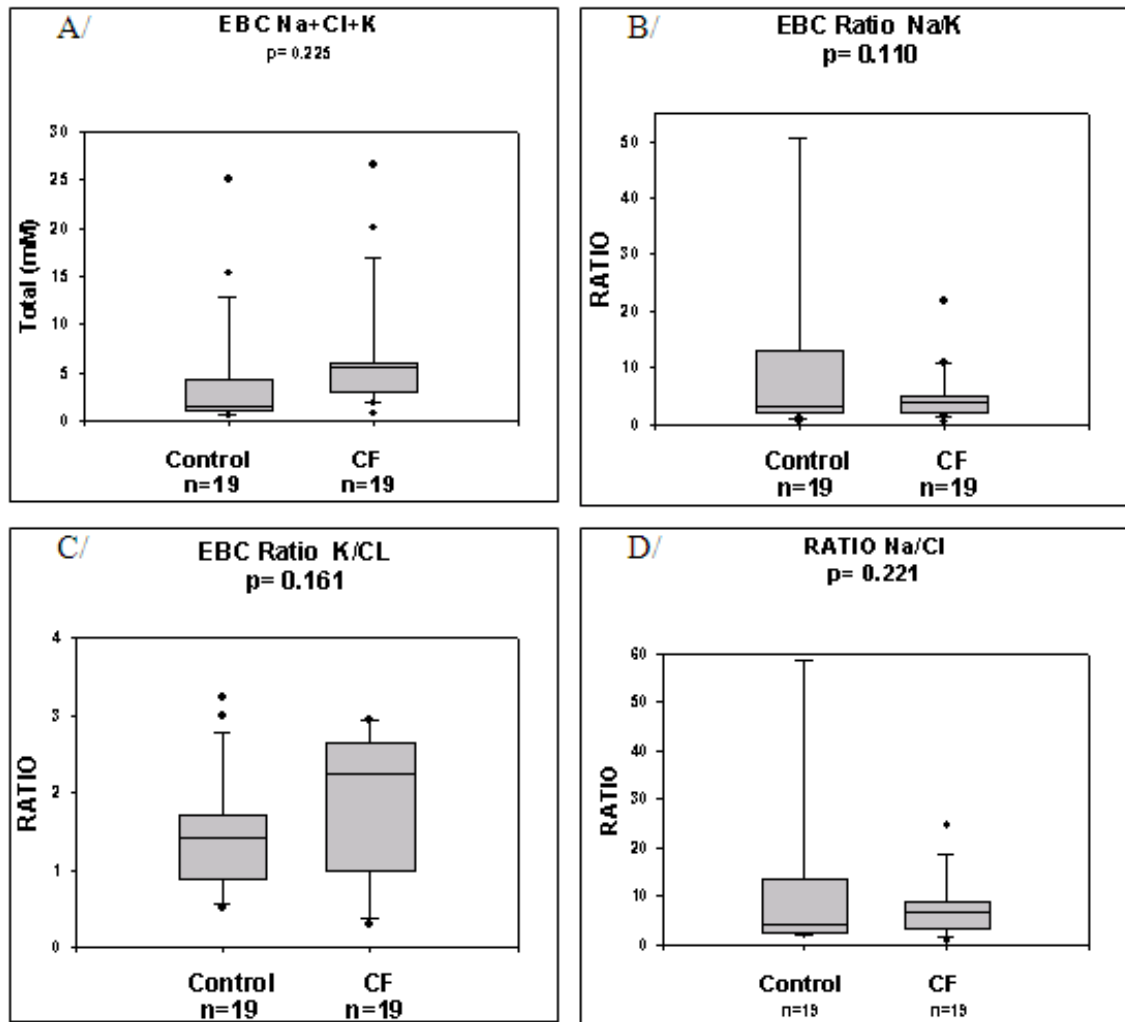


Figure 35/

- A. EBC ion ratios – Total Sodium + Potassium + Chloride - No difference between CF and controls
- B. Ratio of Sodium to Potassium - No difference between CF and controls
- C. Ratio of Potassium to Chloride - No difference between CF and controls
- D. Ratio of Sodium and Chloride in control and CF subjects. - No difference between CF and controls

No difference between controls and CF for ratio of ions Sodium, Potassium and Chloride.

5.4.3 Discussion

No differences in ions or ratios of ions were found. Ranges of ion levels were noted. Sodium and chloride correlated strongly in each individual in both the CF and the control group (Na/Cl $r=0.66$, $p=0.000005$), implying that the assay techniques were successful. Therefore, the levels are likely to reflect the genuine ion concentration in EBC of CF and Non CF subjects. From these data, there is no suggestion that CF EBC (and therefore potentially ASL) contains higher or lower concentrations of salts.

Until now, there has been controversy around whether the CFTR function affects hydration of ASL (hydration hypothesis (Boucher, 2003, Boucher, 2004)) or salt tonicity in ASL (low salt hypothesis (Smith et al., 1996)). According to the hydration hypothesis, in the normal state, sodium controls ASL height via the ion channel ENAC, which in turn is controlled by CFTR. However in CF, ENAC is permanently open, sodium absorption is accelerated into the cell. By osmosis, water and any extracellular chloride would then be absorbed through leaky tight junctions, leading to a depleted isotonic ASL. Therefore in the hydration hypothesis, there should be no difference in the ion composition of CF ASL (and EBC) compared to control subjects. In the low salt hypothesis, it is postulated that the normal ASL is hypotonic, and stems from the finding that CF ASL had higher salt level than control in cell culture (air liquid interface) (Smith et al., 1996). This was explained by CFTR controlling chloride absorption into cells (as well as ENAC controlling sodium) and tight junctions stopping any influx of water. This would lead to an hypotonic ASL. In CF, chloride and sodium absorption would not be controlled and therefore ASL sodium and chloride would be isotonic (by osmosis). Therefore if the low salt hypothesis is correct, we should see more sodium and chloride in the ASL (and EBC) of CF subjects. If EBC is directly sampling ASL (dissolved in exhaled water vapour) then these data are potentially very important and would suggest that the hydration hypothesis is correct (Knowles et al., 1997). This would have particular importance for CF gene therapy as it would imply that tight junctions are leaky resulting in improved transfection efficiency.

EBC data should not be used to comment on whether ASL is hypotonic or isotonic as we do not know the dilution effect of exhaled water vapour.

With regard to the purpose of this thesis, to detect markers of CFTR, it was not possible to distinguish CF from control subject using the measurement of Na⁺, Cl⁻ and K⁺ ions in EBC. Therefore it will be necessary to use surrogate markers of CFTR function rather than direct measures.

Assay of Na⁺, Cl⁻ and K⁺ ions in EBC cannot be used to distinguish CF from non-CF subjects and therefore does not have a role in CF GT trials.

5.5 Analysis of combination of EBC Biomarkers

5.5.1 FEV₁

5.5.1 Introduction

The gold standard non-invasive marker is the lung function marker forced expiratory volume in 1 second. The FEV₁ is the best discriminator used in clinical practice. Its main disadvantage is that it reflects a lifetime of deterioration and damage. This deterioration is multifactorial in causation, reflects age of onset of pseudomonas colonisation, compliance with treatments, social class, and has a poor correlation with genotype. For FEV₁ day-to-day variability is significant (200ml) and it is relatively less sensitive to short term changes in lung inflammation e.g. during infective exacerbation. The FEV₁ of our subjects was measured for comparison and was the gold standard marker to which all biomarkers would be compared.

5.5.2 Methods

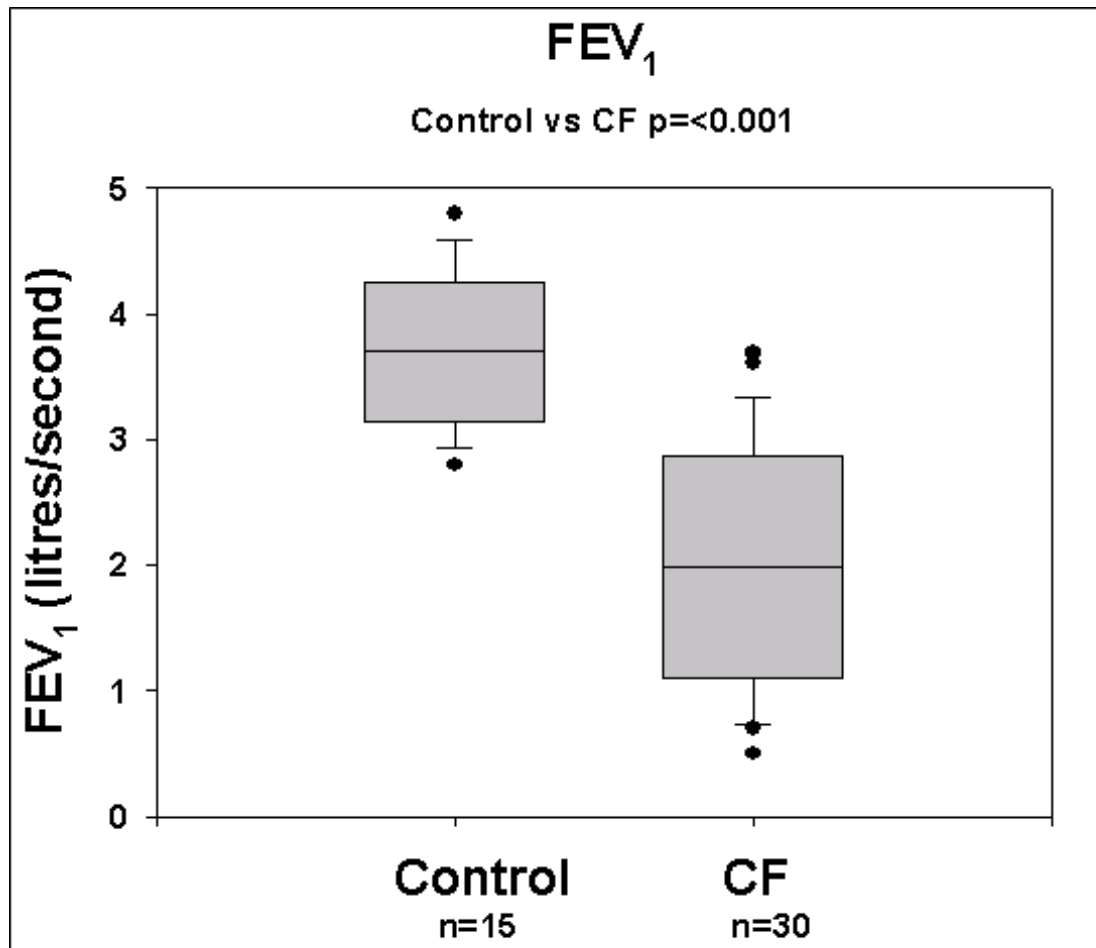
The sensitivity and specificity of FEV₁ can be calculated using tree based algorithms. This was used as a way of assessing how good a discriminator FEV₁ was (of CF). Pattern recognition software was used to calculate the true and false positive and true and false negative predictive value of FEV₁.(CIPHERGEN, USA)

Subjects.

Adults who were either healthy control subjects or CF subjects whilst stable performed spirometry.

5.5.3 Results

Lung function (FEV₁) was significantly lower in CF subjects (n=28, Median 2.0 (IQR 1.1-2.9)) compared to controls (n=15, Median 3.7 (IQR 3.2-4.3)) p<0.001. Using pattern recognition software - the predicted sensitivity was 65% and specificity 95%. See figure 36.



FEV1
Sensitivity - 65%
Specificity - 95%

Figure 36. Lung function parameter – FEV₁ in controls and CF subjects.

FEV₁ is significantly lower in CF subjects when compared to healthy controls, p<0.001.

5.5.3 Discussion

FEV₁ is a good discriminator of CF compared to controls. As FEV₁ drops gradually through a patient's lifetime, it is more likely to reflect a life-long deterioration rather than reflecting well-being in recent weeks and months. For this reason it may be that a significant improvement in FEV₁ will not be attainable by successful gene therapy.

As a minimum target for biomarkers of CF, significance should be $p < 0.001$ and sensitivity at least 65% sensitive, to be comparable to FEV₁.

5.6 Comparison of all markers against FEV₁

5.6.1 Introduction

None of the individual breath markers investigated so far in this thesis gives a sensitivity as high as FEV₁. Therefore it is necessary to combine more than one marker to optimise discrimination. The technique used was to generate a decision tree to optimise discrimination between CF and non-CF subjects. This way all markers could be tested in a combined way.

5.6.2 Methods

A statistical package was used to identify patterns within the data set which could be used to discriminate CF from control groups (Ciphergen pattern recognition software, USA). Sensitivity and specificity were also calculated. A data table of all previous EBC and EB gas data was generated. Complete data sets for the markers eCO, eNO, EBC nitrite, pH and ammonium were collected from adults with CF (n=28) and healthy control subjects (n=15). This dataset represents a mixture of CF subjects who were stable and during exacerbation.

5.6.3 Results

A discriminatory tree was generated which used the EBC markers ammonium, pH, and nitrite as well as eNO (figure 37).

Sensitivity and specificity were calculated as 67% and 92% respectively.

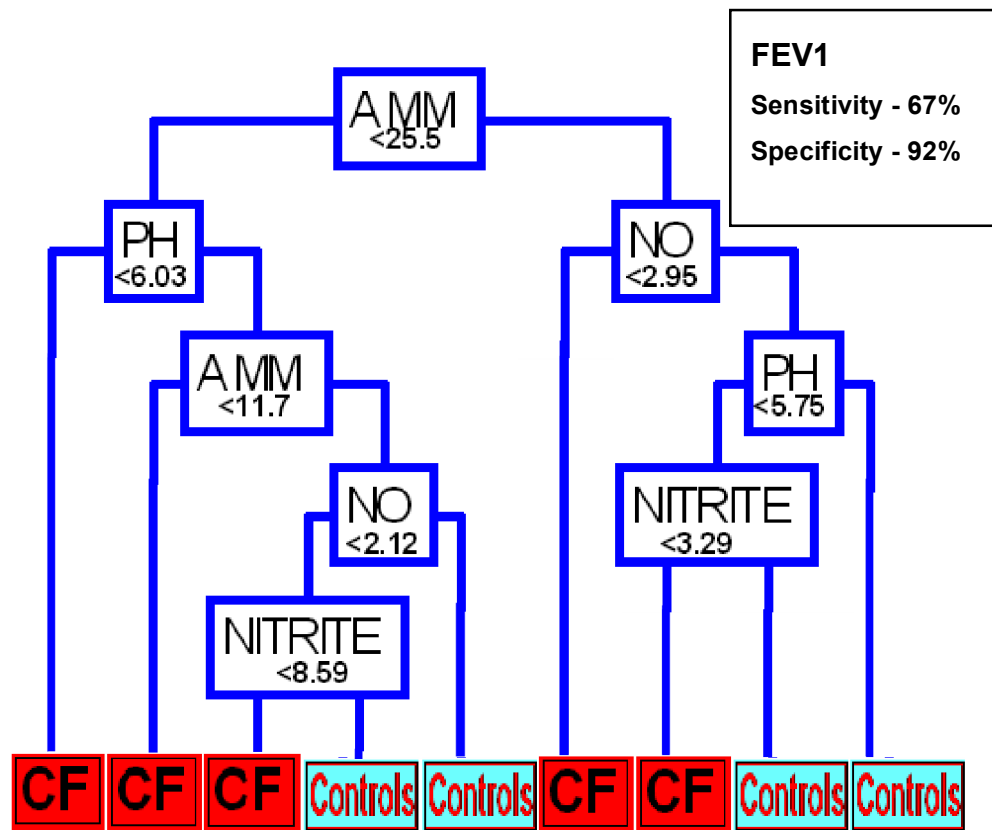


Figure 37. Biomarker decision tree for combination of markers.

Diagrammatic representation of biomarker decision tree. This dendrogram represents the discriminatory levels of a combination of EB gas and EBC markers. Therefore, in a new sample initial discrimination starts with EBC ammonium (at the top). As can be seen from the decision tree, if the ammonium level is less than $11.7\mu\text{m}$ then the sample is likely to be CF. If the ammonium level is not low ($>25.5\mu\text{m}$) but the eNO level is low ($<2.95\text{ppm}$) then the sample is likely to be CF.

5.6.4 Discussion

By combining EBC data, it was possible to discriminate between CF and Non CF better than by using FEV₁. The combined tree showed slightly higher sensitivity and much higher specificity than FEV₁. As these biomarkers are generated over short periods of time, they have the potential reflect short term changes in lung pathophysiology and therefore ultimately may prove superior to FEV₁.

When each marker was analysed individually (using mann-whitney rank sum), the markers which were most different were EBC ammonium and pH (i.e. lowest p values). When a combined decision tree is used, it can be seen that eNO and EBC nitrite (which did not have low p values) are also relevant in subjects who do not have both a low ammonium and a low pH. It is interesting to postulate that pH and ammonium may represent one component of CF lung disease and that NO and nitrite may represent another. The decision tree deems a low eNO and low nitrite as helping discriminate CF from controls, which in itself is surprising as these are thought to be induced by proinflammatory cytokines via inducible nitric oxide synthetase. Perhaps it is not inflammation that they are reporting on, instead it could be through the three mechanisms discussed in chapter 3. These mechanisms are CFTR interaction with iNOS, reduced nitrosothiol (glutathione) secretion or pseudomonal consumption of nitric oxide. All three modes of action can be directly linked to CFTR function but the most significant possibility is that of CFTR interaction with iNOS. If EBC nitrite and eNO are reporting iNOS function, then these assays could be used to report directly on CFTR function i.e they should rise with successful gene therapy.

Whatever the components of CF lung disease that these markers are reflecting, using the decision tree would suggest that the markers which should go forward to use in gene therapy trials are EBC ammonium, pH and nitrite and eNO.

These data show a higher sensitivity to detect CF than FEV₁ (67% vs 65%). As gene transfer efficiency with non-viral gene therapy is low, any anticipated improvements from baseline will be low, therefore ideally markers would have much higher sensitivity and specificity than generated by eNO and EBC markers alone, ideally individually such markers would have sensitivity and specificity values close to 100%.

To validate these data, a further complete dataset (n=20 in each group) should be collected. Then the decision tree could be tested to see if it can genuinely discriminate CF from non-cf subjects.

It would be possible to utilise this technique to identify the most important markers of inflammation in CF subjects by prospectively following them through an exacerbation i.e. collected data at time of exacerbation and then again once they had improved back to stability. Then these datasets could be compared using the software to tease out the best markers of inflammation.

6.0 – Proteomics techniques

6.0 – Proteomics techniques – All sample types

6.0.0 Introduction

After completion of work validating known/established markers, a technique to find novel biomarkers was used. As EBC Total Protein concentration was raised in CF subjects, a proteomics approach was chosen to identify the most important proteins.

This approach involved the global study of proteins within Exhaled Breath Condensate, Bronchoalveolar Lavage fluid and Induced Sputum. The technique employed was mass spectrometry with further protein identification using polyacrylamide gel electrophoresis coupled with mass spectrometry.

6.1 Surface Enhanced Laser Desorption Ionisation (SELDI)

Surface Enhanced Laser Desorption Ionisation Time of Flight (SELDI TOF) is a mass spectrometry technology supplied by Ciphergen Biosystems (PBS 2C, Ciphergen, USA). It offers a coupling of “Surface Enhancement” of proteins using on-chip chromatographic separation by their chemical class with “Laser Desorption/Ionisation Time of Flight” (as with matrix assisted laser deionisation time of flight - MALDI TOF) technology.

6.1.0.0 Surface Enhancement

Biological samples are applied to different chip surfaces and allowed to bind (figure 38), then non-bound components are washed off. This allows separation of the proteins into different chemical classes and is important as biological fluids are complex, containing a large number of high abundance proteins and less abundant proteins may therefore not be detected. Less abundant proteins are not detected using SELDI due to a saturation of the mass spectrometry signal by high abundance proteins (e.g. albumin) and also by interference of non-protein compounds e.g. salts and DNA.

Different chromatographic surfaces allow binding of different proteins reflecting their chemical characteristics. These surfaces are Cationic, Anionic, Hydrophobic, Metal Affinity and Normal Phase (Global binding) chip surface (figure 39).

6.1.0.1 Laser Desorption/Ionisation

SELDI (and MALDI) mass spectrometry platforms use a “matrix” to donate protons to proteins in the sample. The matrix and proteins are dried onto a metal chip and this chip is then inserted into the instrument. A vacuum is generated along a time of flight tube (1m long in the case of SELDI). A laser is used to vaporise the sample and then the protonated sample is pulled towards the cathode. As small proteins travel faster, they hit the detector first, allowing separation of proteins by mass and charge (MZ^+). Medium sized proteins hit the detector slightly later and give a stronger signal (due to higher momentum). As larger proteins hit the detector later (after detector has reached close to saturation), they elicit a smaller signal (figure 40). In theory, proteins between 500Da and 500kDa can be detected, with peak sensitivity between 2kDa and 20kDa. The data generated can be viewed in three different ways – spectral view, gel view or data view. (Figure 41)

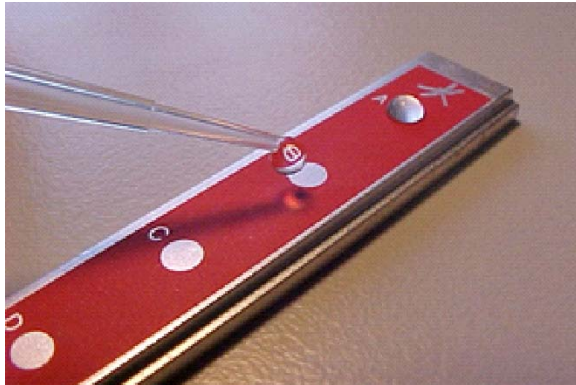


Figure 38 – Application of sample to SELDI chip surface.

Sample being applied to SELDI chip surface.

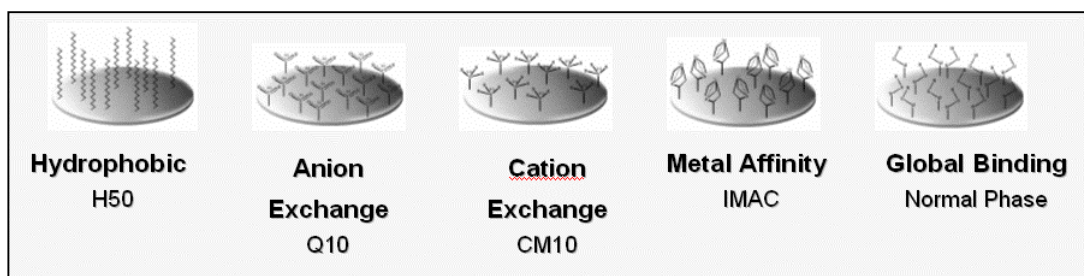


Figure 39 – Chip surfaces used in this thesis.

The five chip surfaces available for experiments enriched for hydrophobic proteins, positively charged (anion exchange) and negatively charged (cation exchange) protein, binding to specific metals (metal affinity) and non selective (global) binding.

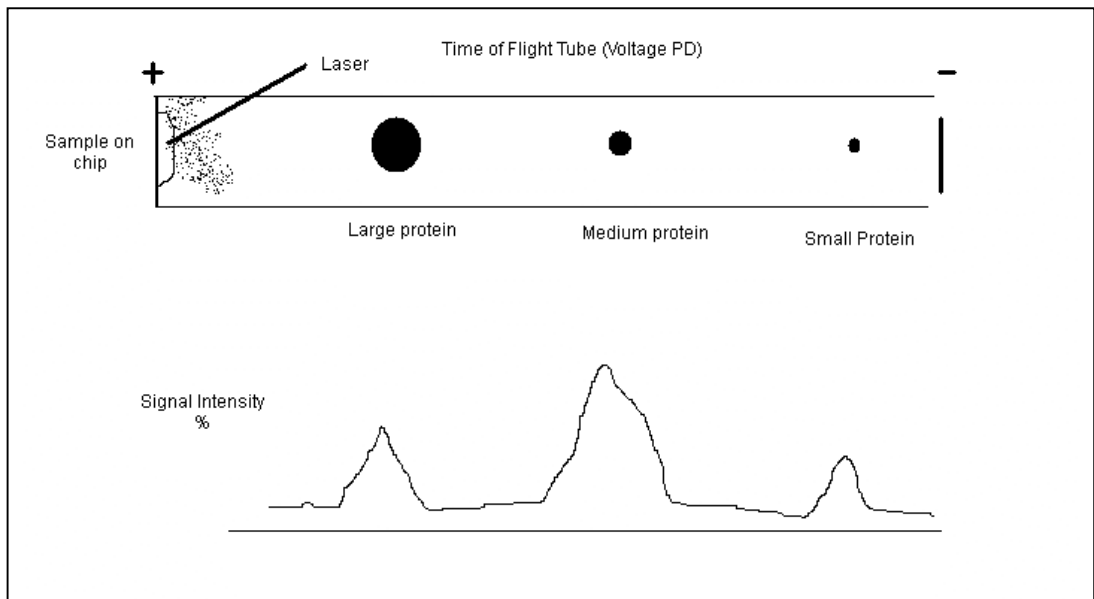


Figure 40 – Diagram demonstrating time of flight and signal intensity generated for different sized proteins.

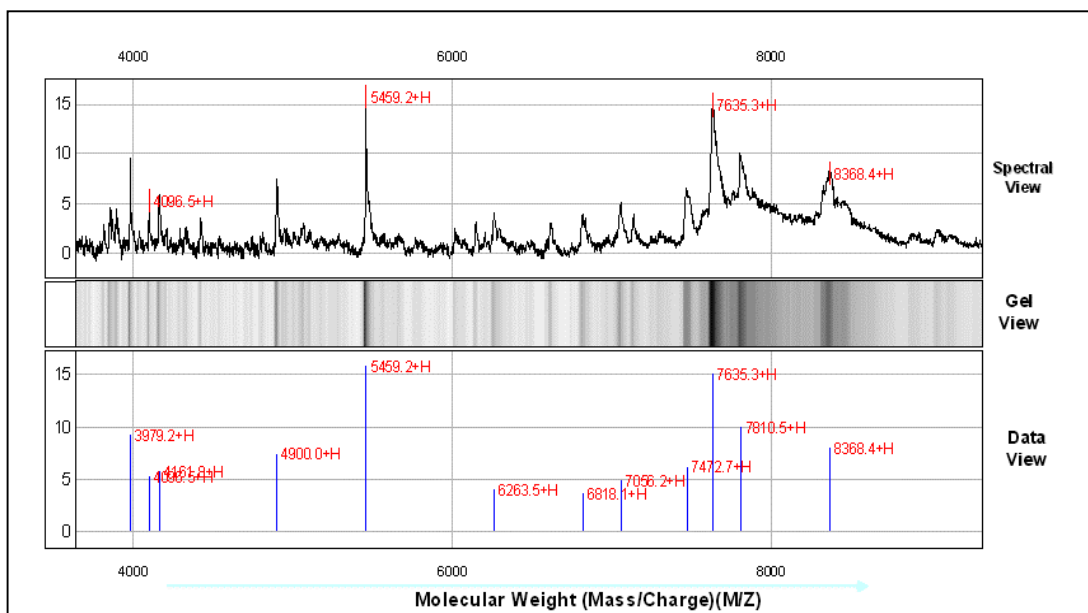


Figure 41 – SELDI software generated data views

The actual data generated can be shown as a spectral view (top), as a computer generated gel band view (middle) or once the data has been extracted from background noise, as a data view (bottom).

6.1.1 SELDI – Potential Chip/binding buffer combinations

As well as different chromatographic properties of each chip, adjustments to binding conditions can be made. The optimal combinations were screened using BAL and EBC fluid. Twelve optimal combinations were identified.

6.1.1.2 Optimised chip/binding buffer conditions

The protocols for each of these binding and washing conditions were developed through multiple experiments using BAL and EBC. The different chip surfaces allow separation due to differencing chemical characteristics of the proteins.

Cationic (CM10) and Anionic (Q10) chip surface

- CM10 at pH4, pH6 and pH8
- Q10 at pH6, pH8 and pH10

Global Protein Binding Surface

- NP20

Hydrophobic

- H50 at low acetonitrile elution (10%)
- H50 at high acetonitrile elution (40%)

Metal Affinity

- IMAC Nickel, Copper and Cobalt.

6.1.3 SELDI Protocols

Bioprocessor versus on spot analysis

The bioprocessor is a funneling system where a larger volume of sample can be added to the chip surface. It performs best due to widespread binding across the chip surface by avoiding a meniscus (avoids reduced binding at chip perimeter). It can be used for larger sample volumes (e.g. >20 μ L). If sample volume is low and its protein concentration is low, it is preferable not to dilute the sample before binding to the chip surface. In this case the sample should be applied directly to the chip surface. Maximum volume for direct application is 7 μ L.

Energy Absorbing Molecules (Matrix)

Two matrices are available for use with SELDI:- SPA – sinapinic acid and CHCA – alpha cyano hydroxy cinnaminc acid. The quality of data (signal intensity) was better with SPA than CHCA using test samples of EBC, BAL and induced sputum, therefore SPA was used. SPA was reconstituted with 50% acetonitrile and 0.5% trifluoroacetic acid (400 μ L per eppendorf).

6.1.4 SELDI data acquisition

6.1.4.1 Deflector Settings

If the signal generated is very strong, this can lead to saturation of the detector of the instrument, which would decrease sensitivity. The matrix used to protonate the sample, can produce a signal below 2kDa. Such small mass protonated compounds can be “deflected” away from the detector to improve sensitivity. The instrument contains a mass deflector for this purpose.

Experimentation revealed improvements in signal with deflector set at 20Da for small peptides (i.e. less than 10kDa) and 2500Da for higher mass proteins (i.e. greater than 10kDa).

6.1.4.2 Analysis Settings

Variations between SELDI instruments require that each has to be individually calibrated to optimise the signal strength. To do this, BAL fluid was assayed after being bound to a CM10 chip at pH6 and the settings which achieved best signal strength were used. These were a digitiser rate of 500MHz and a deflector voltage of 2650 V.

Signal to noise data filtering.

The data generated shows a varying baseline (background noise) and genuine protein signals (peaks) within it. To analyse and extract the data, filtering of baseline noise is necessary. Indeed early publications which did not adequately perform baseline filtering, were heavily criticised because some of the “biomarkers” discovered were actually baseline noise (Petricoin et al., 2002). Guidance was obtained from the manufacturer, who recommended using a signal to noise ratio of at least 3 for peptides of less than 10kDa and of greater than 5 for proteins larger than 10kDa). These settings were confirmed as detecting genuine signal by checking all data by eye to ensure adequate signal detection occurred.

6.1.5 SELDI Data Analysis

6.1.5.1 CIPHERGEN Peaks

This statistical package was used to analyse the crude data, and was used to identify peptides and proteins. The main process to do this was to identify peaks from the background noise (using signal to noise ratios). As explained above, previous work using the SELDI instrument was criticised because data were extracted and analysed from areas below limit of detection of the equipment (Petricoin et al., 2002). To avoid such problem, we set peak detection limits on our data set. Peptides (i.e. <10kDa) with a signal to noise ratio of greater than three were considered likely to be genuine and proteins (i.e. >10kDa) with a signal to noise of greater than five were considered likely to be genuine. These settings were recommended by Ciphergen representatives, and were monitored throughout all experiments by checking a subset of the data by eye. These extracted data were then applied to other statistical packages for further analysis.

Variation in the quantity of protein in different samples leads to a different strength of signal. To compensate for this, the data can be “normalised” to total signal (defined as area under the curve) so that all data are comparable. To identify robust biomarkers, strict normalisation criteria were adopted. This effectively normalised all samples to their total protein content, and therefore total signal was used as an internal standard for all data produced.

6.1.5.2 Ciphergen Biomarker Wizard

Using the data extracted from Ciphergen peaks, samples were then unblinded and coded into disease groups. The software allowed identification of the biomarkers most different between groups. Comparison was made using Mann Whitney Rank Sum. Significant difference was deemed as having a p value less than 0.001 at this stage.

Biomarkers which were present in at least 15% of all spectra analysed were chosen for differential expression analysis, with a first pass screen of data using a signal to noise ratio of 5:1 and a second pass of analysis was applied with a signal to noise of 3:1.

6.1.5.3 Ciphergen Express

This software allowed comparison between data sets (i.e. between subject groups and chip surfaces). Data analysis settings were applied at same settings as using Ciphergen Peaks and Biomarker Wizard software to ensure data generated were not different.

6.1.5.4 Ciphergen Biomarker pattern recognition software

This package was used to give an estimate of the sensitivity and specificity of each test. After data collection and analysis, this software can be used to combine multiple markers to generate a decision making tree to improve the sensitivity and specificity by combining different markers.

6.2 Exhaled Breath Condensate – SELDI analysis

6.2.1 Introduction

SELDI TOF allows the identification of large numbers of proteins in a biological sample, based on molecular weights and chemical characteristics. It avoids the limitations of antibody binding and of only analysing predetermined proteins. It is able, therefore, to identify proteins not previously appreciated to be potentially valuable biomarkers. The technology has been applied to serum and urine to identify disease specific biomarkers (Petricoin et al., 2002, Rogers et al., 2003, Seibert et al., 2004) but its application to BAL fluid allows a valuable evaluation of proteins in the areas directly involved in airways inflammation.

Exhaled breath condensate contains 0.5-25µg/ml of protein (see chapter 3). SELDI chip surfaces require a protein concentration of at least 5µg/ml to bind to the surface to generate reproducible data (5µg/ml concentration recommended by Ciphergen).

Therefore the concentration of protein EBC is at the lower limit of detection for SELDI technology, even though SELDI is considered to be at least 1000x more sensitive than ELISA.

6.2.2 Methods

To concentrate the sample freeze drying was performed. As low sample volumes could be added to the chip surface (e.g. 5 µl), 1ml of EBC could be concentrated 200 fold. This would imply that the protein concentration would be a minimum of 100mcg/ml in 5µl of concentrated EBC, and should therefore be sufficient for binding to the chip surfaces.

Initial data using EBC at a demonstration by Ciphergen were encouraging, albeit in a small dataset (n=4 CF vs n=4 non-CF). However, once the instrument was purchased, it was not possible to reproduce the initial data.

Indeed reproducibility of any data generated was infrequent. As reproducibility is a vital component of any clinically useful test, this negated further research on EBC.

Contamination issues.

Part of the difficulty with reproducing initial results was that low levels of contamination were found to generate a signal. It was realised that the pipette tips used for the experiment were a source of contamination as the tips used were from general laboratory stock and were racked by hand.

The tips were changed to preracked, proteomic grade tips and this reduced the frequency of contamination being seen.

A further source of contamination was from the laboratory source of water. Initial experiments used an ELGA ultrapure water filtration system. Despite frequent servicing and changing of the filters, this water source contributed to contamination using SELDI. The water source was changed to Fluka ultrapure water.

6.2.3 Results

Once the changes to resolve contamination issues had taken place, this caused a significant reduction in the number of proteins detected in EBC. As techniques to avoid contamination improved within each part of the protocol, the number of proteins detected fell. Once we had removed these potential sources of contamination, the number of proteins detected fell to zero (figure 42)

Sporadically a signal was detected with a mass spectrometry profile similar to BAL but this had occurred in less than 1 in 20 samples, as shown below (figure 43). The peaks below have been identified as the human alpha defensins 1, 2 and 3 by previous researchers (Zhang et al., 2002). A later publication retracted the source of the alpha defensins in their published paper, commenting that the source of the alpha defensins may have been contamination – in this case from neutrophils (Zhang et al., 2004).

6.2.4 SELDI EBC – Discussion

After contamination issues were improved, we could not generate reproducible data from EBC despite confidence that we were within the acceptable range of protein concentration for the instrument. Therefore it was concluded that no proteins could be detected with highly sensitive equipment. It is conceivable that data generated from all EBC work reflects contamination and further work to investigate this is warranted.

As only 1 in 20 samples generated acceptable data the decision was made to change the focus from EBC onto the proteomic analysis of induced sputum and bronchoalveolar lavage fluid for development of novel biomarkers of inflammation and CFTR function.

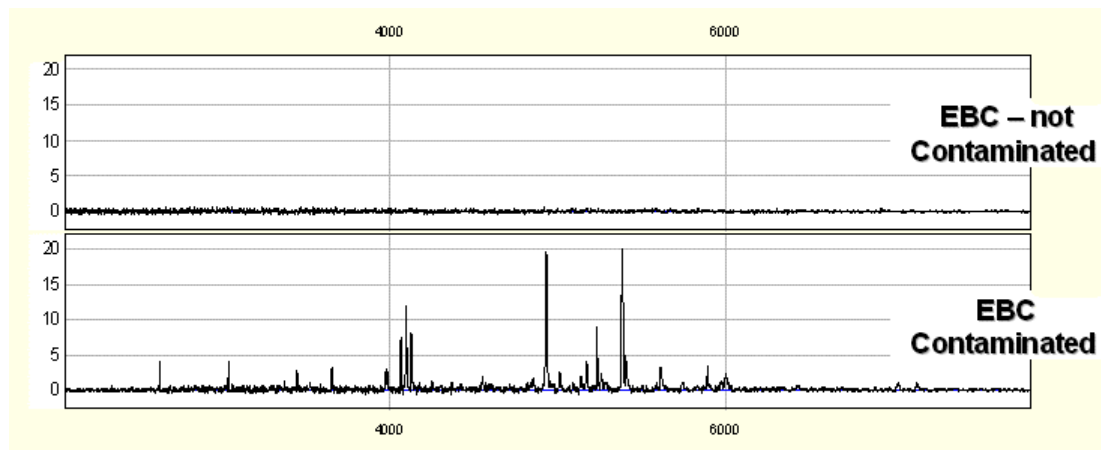


Figure 42. Contamination evident in EBC

This figure shows two samples of EBC. The top graph represents data generated after using proteomic grade pipette tips and proteomic grade ultrapure water. The lower graph shows data where suspected contamination generated a protein signal.

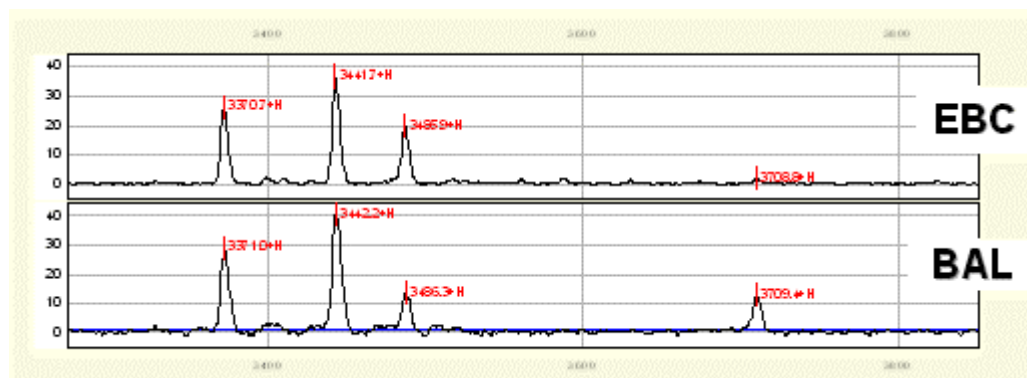


Figure 43. Similar profiles of EBC and BAL

These data show similar profiles of EBC and BAL. These data were sporadic in nature and found not to be repeatable. The three peptides shown to the left are human alpha defensins 1, 2 and 3 (Zhang et al., 2002).

6.3.0 Effect of EBC on recombinant Interleukin 8.

From chapter 5, EBC seemed to have a deleterious effect on the detection of interleukin 8 using ELISA. SELDI has superior sensitivity at the mass range of cytokines and chemokines. Therefore, we used SELDI to assess whether there was an effect on recombinant interleukin 8 by EBC.

6.3.1 Methods.

EBC was collected from one healthy control adult, freeze dried and reconstituted 10 fold as per techniques used in chapter 5. 10 pg of recombinant interleukin 8 (Biosource, Belgium) was reconstituted in ultrapure water and then added to either 10 fold concentrated EBC or ultrapure water (as a control). The solutions were then applied to a CM10 chip at pH 6.

6.3.2 Results.

Recombinant Interleukin 8 was altered by EBC and not by ultrapure water. A mass shift of +251.3 Da was seen when EBC was added to the recombinant interleukin 8 (see figure 44).

6.3.3 Discussion

A mass shift of 251 Da could represent many different protein modifications by EBC (see table 14). The modifications listed in Table 14 could all be with the mass accuracy error at this molecular weight (mass accuracy reported to be 300 parts per million with SELDI, which is significantly less than MALDI).

Palmytolation is a protein modification which could theoretically occur in the present of EBC, as BAL is comprised of significant quantities of palmitoyl attached to surfactant protein C, and it is the loss of the palmitoyl which confers SP-C activity to form amyloid sheets (during inflammation) (Gustafsson et al., 2001).

This interesting observation could give an explanation for the interleukin 8 EBC data generated using ELISA. Palmytolation (or another protein modification) of recombinant interleukin 8 by EBC would have the potential to mask the epitopes available for ELISA detection leading to “decay” of inteleukin 8 signal with time.

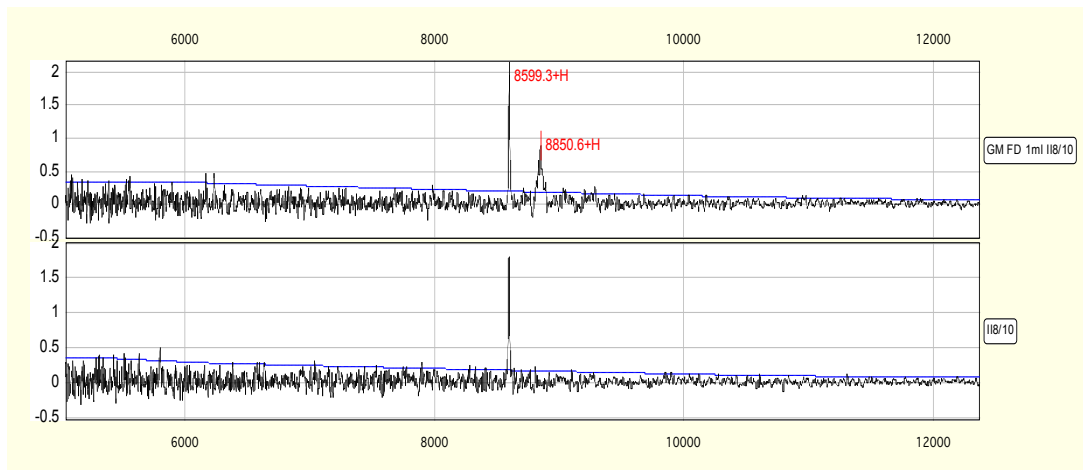


Figure 44. Mass shift effect of EBC on recombinant interleukin 8.

These data show the effect of EBC on recombinant interleukin 8 as assayed using SELDI. The upper graph shows EBC with an interleukin 8 spike and the lower sample shows recombinant interleukin 8 spiked into water as a control. In the upper graph a second smaller peak is seen with a mass shift of +251.

Mass Shift	Protein Modification
238	2-(p-biphenyl)isopropyl-oxycarbonyl (Bpoc)
238	Palmitoylation
242	Triphenylmethyl (Trityl, Trt)
243	Tyrosinyl Sulphate
243	Phospho Tyrosinyl
252	Pbf (pentamethyldihydrobenzofuransulfonyl)
252	3,5-Diiodination (of Tyrosine)
258	a-N-6-Phosphogluconoylation (His Tagged proteins)
259	N alpha -(gamma-Glutamyl)-Glu2
266	O-GlcNAc-1-phosphorylation (of Serine)
266	Stearoylation
266	Pmc (2,2,5,7,8- Pentamethylchroman-6-sulphonyl)
272	Geranylgeranylation

Table 14. Post translational modifications at mass range 238-272

This table shows the mass shift effect on a protein of the different post translational modifications in the mass range 238-272 Da. The mass shift effect seen on interleukin 8 with EBC was +251 Da and with a mass accuracy of 300ppm, the modification could potentially be any of these modifications.

6.4 SELDI - BronchoAlveolar Lavage analysis

Samples for this study were kindly supplied by Dr Tom Hilliard as part of the UKGTC collaboration.

CF lung disease commences soon after birth. It is not yet known if the basic defect precludes to an inflammatory defect or to invasion by infecting organisms. To validate non-invasive markers, it is appropriate to identify them in ASL where possible. Due to the progressive nature of CF, generally adults have more severe lung disease, making BAL procedures potentially unsafe. Therefore BAL procedures are avoided as much as possible in the adult CF population. However, in children BAL is performed as part of CF (and non-CF) patients routine care. They are not performed in healthy children.

The use of sample material from early in life could potentially have advantages for the purposes of this thesis, in that infection and inflammation may be present to a lesser extent than adults and may therefore reveal surrogate markers of the basic CF defect as well as inflammation. As the control group had non-CF lung disease, there was also the potential for identification of CF specific markers of inflammation.

6.4.1 BAL Methods

6.4.1.1 Subjects

Subjects were children undergoing flexible bronchoscopy, for clinical reasons, in a single paediatric centre. Bronchoalveolar lavage (BAL) was performed in 39 children with CF and 24 non-CF children with a range of other respiratory diseases [lower respiratory tract infection (n=11), chronic cough (8), primary ciliary dyskinesia (7), croup (3), others (9)]. The mean age of CF subjects was 7.0 years (0.3-14.8y) and non-CF subjects 6.3 years (0.4-15.3y). The reasons for bronchoscopy in the CF children were as follows: to detect bacterial infection after diagnosis of CF (8 cases), failure to respond to antibiotic therapy during a pulmonary exacerbation (24), lobar consolidation (5), microbiological surveillance while undergoing a surgical procedure (2).

6.4.1.2 BAL processing

Flexible bronchoscopy was carried out under general anaesthesia, with BAL performed in the middle lobe using 3 aliquots of 1ml/kg of normal saline. The return fluid was

pooled. BAL fluid was centrifuged at 2000g x 10min to separate cellular and fluid phases. The supernatant was stored at -80°C until analysis. The cell pellet was resuspended, treated with 0.1% dithiothreitol and differential cell counts performed on a cytopsin preparation stained with May-Grunwald-Giemsa. IL-8 concentrations were measured by commercial ELISA (R&D, Minneapolis, USA).

6.4.3 Results

1277 proteins, >4kDa, were detected using 12 different surfaces and binding conditions. 202 proteins identified were differentially expressed in the CF samples ($p < 0.001$). 167 of these were over expressed and 35 were under expressed. When Bonferroni correction and removal of poorer signals was applied, 59 proteins were differentially expressed. The platform software modelling analysis of these proteins gave a $\geq 90\%$ sensitivity and specificity for CF compared to non-CF subjects. Statistically, the most significant biomarker had a predicted mass of 5.163 kDa (Figure 45.1). This biomarker completely discriminates CF from control and most efficiently on the IMAC nickel chip surface. 6 example proteins are shown (figure 45).

6.4.3.1 Bonferroni Correction

By applying bonferroni correction for multiple comparisons (of total 1287 proteins), the significant p value would be > 0.000038 . Using this correction, there are 59 proteins in CF BAL which are different. Whilst this correction reduces the chances of a type 1 error, it raises the chance of a type 2 error, meaning that potential biomarkers could be lost by applying this correction. As we want robust biomarkers, which would discriminate a 5% improvement with gene therapy, bonferroni correction was applied.

6.4.3.2 Removing weaker signals

Initial screening of data to remove weak signals was performed by applying settings to detect signal/noise ratio > 3 and enabling the detecting peaks in $> 20\%$ of all samples. In

spite of this, weak signals were still present. To remove weak signals, data with a mean signal intensity of less than 4 were removed from analysis. As the mass deflector was set to 4 kDa (for mid mass data recording), proteins below this were removed. These steps further reduced the number of different proteins from 59 to 23.

6.4.3.3 Duplicate peaks (present on more than 1 surface)

Some proteins were present on more than one chip surface. A mass accuracy of , 600 parts per million is appropriate for SELDI, therefore if >1 protein was present within a 0.0006% mass range, the less significant was removed. This procedure identified 2 duplicates – 5165 IMAC Nickel (and 5167 CM10 pH6) and 64929 Q10 pH10 (and 64961 Q10 pH6). This step therefore reduced the number of proteins from 23 to 21 (Table 15). The 6 proteins with highest significance are shown as examples (Figure 45).

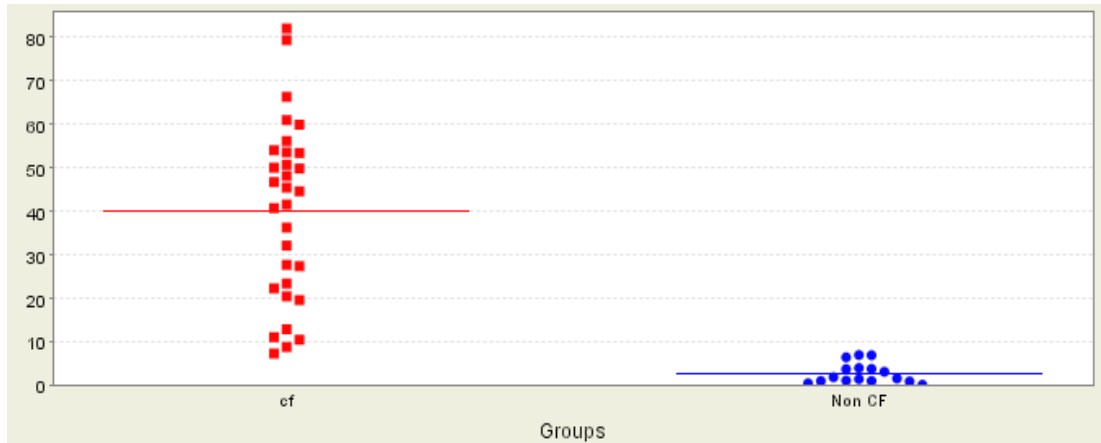
6.4.4 SELDI BAL Data – 21 proteins with lowest p values

Rank	Chip Surface	Protein Mass (Da)	p value
1	IMAC Nickel	5165	0.00000003
2	IMAC Nickel	10623	0.00000139
3	Q10 pH 10	9594	0.00000276
4	Q10 pH 6	10361	0.00000426
5	Q10 pH 6	10380	0.00000484
6	Q10 pH 6	7507	0.00000626
7	Q10 pH 6	7552	0.00000711
8	Q10 pH 8	10611	0.00000752
9	Q10 pH 6	32665	0.00000916
10	IMAC Nickel	5323	0.00000974
11	CM10 pH 4	7266	0.00001129
12	Q10 pH 10	5041	0.00001175
13	Q10 pH 6	10336	0.00001175
14	Q10 pH 10	6968	0.00001620
15	CM10 pH 6	10589	0.00001964
16	Q10 pH 6	9651	0.00002756
17	CM10 pH 8	5031	0.00002771
18	IMAC Nickel	5300	0.00002969
19	Q10 pH 6	8687	0.00003104
20	Q10 pH 6	8879	0.00003104
21	Q10 pH 10	64929	0.00003366

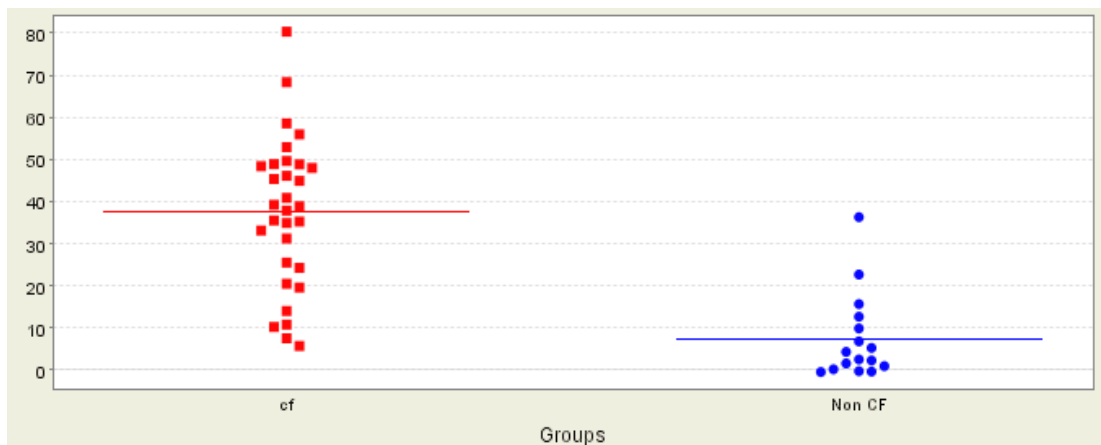
Table 15 – Lead 21 proteins in BAL which were different between CF and control subjects

6.4.5 Figure 45. 6 examples of the 21 BAL Biomarkers of CF lung disease.

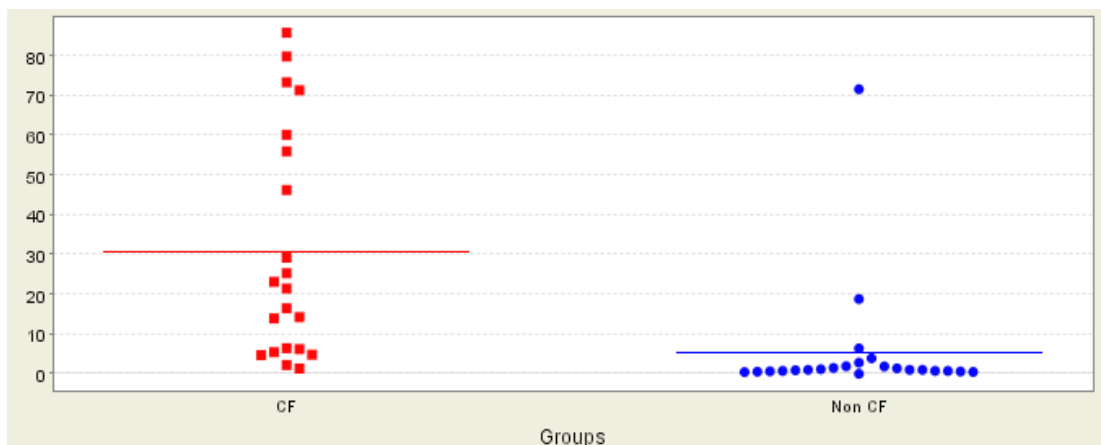
1 - IMAC Nickel – 5165Da, p=0.00000003



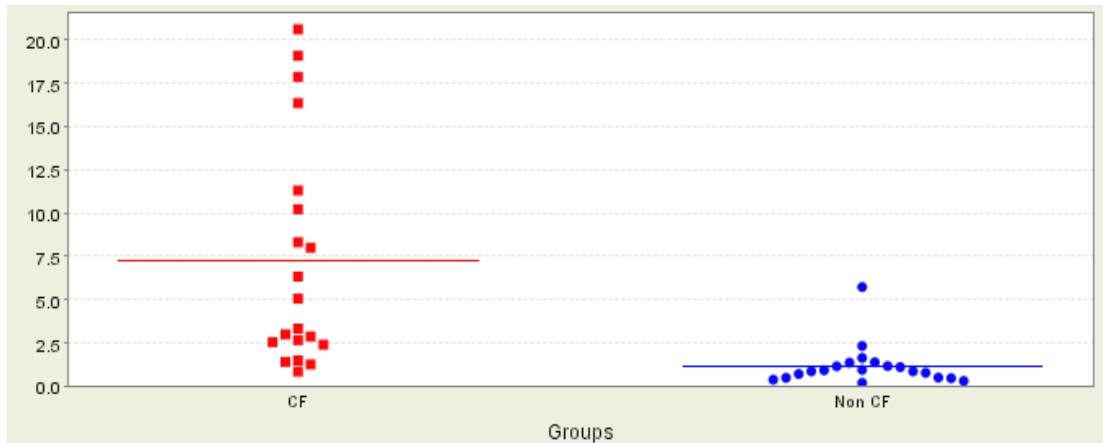
2 - IMAC Nickel – 10623 Da, p=0.00000139



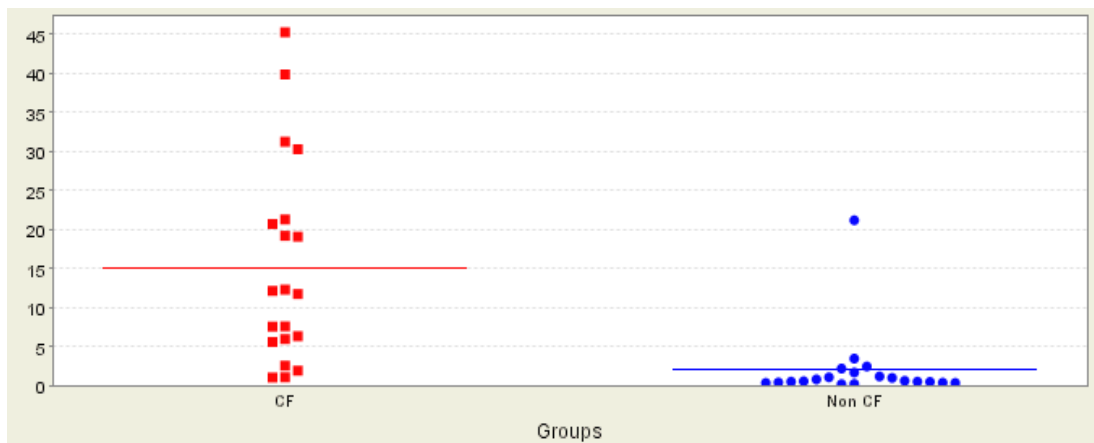
3 - Q10 pH 10 - 9594 Da, p=0.00000276



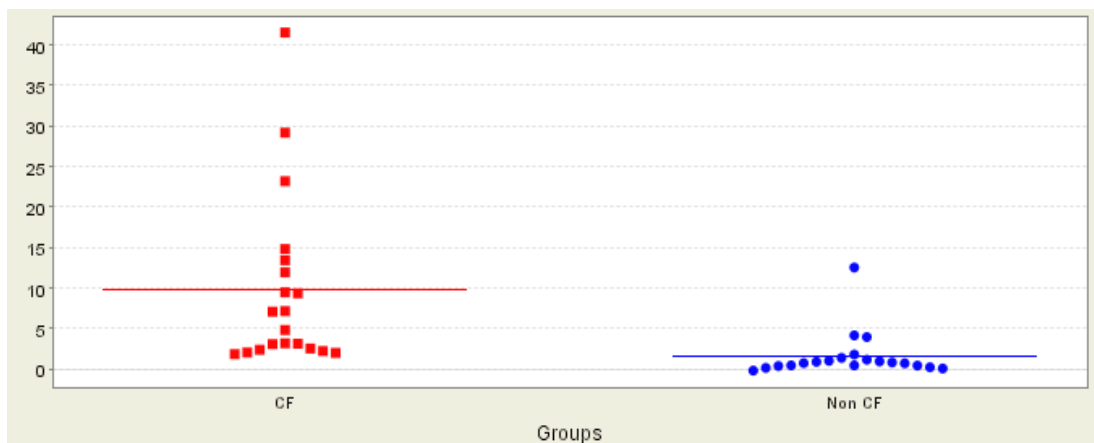
4 - Q10 pH 6 - 10361 Da, p=0.00000426



5 - Q10 pH 6 - 10380 Da, p=0.00000484



6 - Q10 pH 6 - 7507 Da, p=0.00000626



6.4.6 Discussion

SELDI TOF analysis of BAL generates a large panel of protein biomarkers that differentiate, with high statistical significance, CF lung disease from a disease control population. While these biomarkers may prove useful in monitoring CF airways inflammation and possibly CFTR function, longitudinal data are required for evaluation of robustness and value of individual proteins.

Even with the application of very restrictive parameters on data analysis (in order to avoid excessive claims of significance), 21 potential biomarkers have been found. These require specific identification. Restrictive limits were applied to the data in order to avoid making unsustainable claims about the number/value of biomarkers thus identified.

Until the biomarkers have been identified by sequencing, their usefulness will not be known. They may give insights into the pathophysiology of the development of early Cystic Fibrosis lung disease. Undoubtedly some of the markers will also represent CF inflammation and it can be anticipated that the markers would consist of cytokine and chemokine signalling proteins, markers of innate and acquired immunity, killing/defense proteins and potentially other markers of CF lung disease.

The protein 5165 was completely discriminatory in these samples taken from children and it is hoped that if this is also true in the adult population. Identification of this protein would clarify its function and whether it is more likely to reflect inflammation or CFTR function.

The list of biomarkers generated will be useful independently as they reflect CF specific aspects of inflammation, and will also be useful for comparison with induced sputum data generated in adults with CF when compared to healthy controls.

6.5 Induced Sputum – SELDI analysis

6.5.1 Introduction

After identification of potential proteomic biomarkers in BAL of children, induced sputum of adults was analysed to investigate whether these markers were detectable non-invasively. There are two components to induced sputum, as with BAL, the soluble phase and the cell phase. As it has been established that the cellular phase of CF induced sputum contains high but variable quantities of neutrophils and healthy control samples of induced sputum contain a larger proportion of macrophages, it is likely that proteomic analysis of the cell phase will simply reflect differences in cell count. It was hypothesised that the soluble phase would include cytokine and chemokine signalling proteins, markers of innate and acquired immunity, killing/defense proteins and potentially other markers of CF lung disease.

6.5.2 Methods

Induced sputum samples were collected from CF adults using the techniques described in chapter 2 and proteomic analysis was performed on its soluble phase.

Initial validation work was performed on samples from healthy controls to ensure that data quality was satisfactory. The quality of data appeared very similar to that of BAL samples analysed. As the hypothesis of this thesis was that non-invasive samples could be used as a replacement for invasive samples, induced sputum and bronchoalveolar lavage fluid were compared. As induced sputum samples were collected from adults and BAL samples from children, it was unfortunately not possible to analyse BAL and induced sputum from the same individual.

6.5.3 Results

Initial test results revealed a large number of proteins detected on the majority of chip surfaces. The only exception being that very few hydrophobic proteins were detected using the hydrophobic chip surface H50.

202 proteins were differentially expressed in the CF samples ($p < 0.001$) using 12 different surfaces and binding conditions. 167 of these were over expressed and 35 were under expressed. After bonferroni correction was applied, there were 76 proteins

which were differentially expressed. Of these a further 16 were removed because of weak signal, leaving 60 proteins.

Of the remaining 60 proteins, 22 were duplicates and 38 proteins were differentially expressed in the CF group. The 38 proteins are listed in table form (table 16). Six example biomarkers (of 38) were represented graphically (figure 46)

6.5.4 Discussion

Data from induced sputum and BAL appeared comparable despite the induced sputum coming from adults and the BAL from children. This is an important observation in that, should SELDI be useful for identifying biomarkers of inflammation and CFTR function, it is possible that these markers could be detected both in induced sputum and BAL.

The 38 biomarkers were found on five different chip surface/chemical combinations (table 16). As with the BAL data, each marker could be used independently to assess response to gene therapy. The markers detected were highly discriminatory between the CF and Non CF group. The analysis criteria used were rigorous in that a type 2 error may have occurred – meaning potential biomarkers have been missed, however this decision was made to ensure than efforts to identify biomarkers (see later in chapter) were targeted to abundant proteins which were highly different in CF and control population.

These biomarkers could be used either once each protein has been identified/sequenced (as an immunoassay) or without identification using SELDI as an endpoint in future gene therapy trials.

Further analysis of this large proteomic dataset is warranted and it is hoped that correlation of clinical characteristics such as CF subjects gene defect, lung function parameters and bacterial colonisation status would be seen. Multivariate analysis should be performed to see if there are patterns of upregulated proteins. Once identified these protein patterns may give information on pathogenic biological pathways.

6.5.5 Combined data - BAL and Induced Sputum Biomarkers

When the BAL and IS data were compared 8 of the 21 lead proteins found in BAL were also present and differentially expressed ($p < 0.001$) in induced sputum. They had MW 10589, 5165, 10623, 9594, 10380, 7507, 10611 and 5300Da. However, in induced sputum, of these 8 proteins, only 3 were abundant and highly upregulated in both BAL and induced sputum (i.e. after bonferroni correction and removal of weaker signals – present in both the 21 BAL markers and the 38 biomarkers). These were proteins of MW 10589, 10611 and 5300 Da.

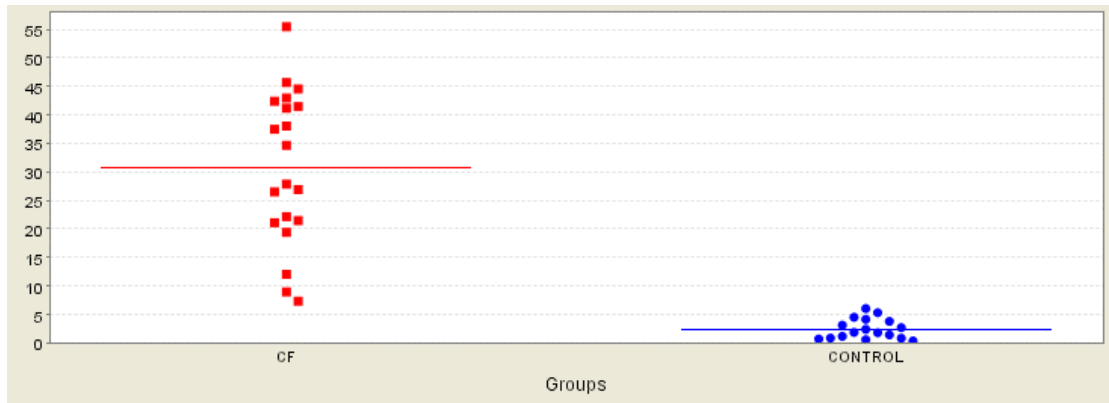
The five markers which were present and upregulated in induced sputum ($p < 0.001$) 5165, 10623, 9594, 10380 and 7507Da were removed after application of bonferroni correction. It would seem plausible that a type 2 error has occurred, and that these 5 markers should also be considered potential biomarkers of CF lung disease.

Rank	Chip Surface	Protein Mass	p value
1	CM10 pH6	10589	0.00000022
2	CM10 pH4	6340	0.00000031
3	CM10 pH6	10187	0.00000036
4	CM10 pH4	5559	0.00000060
5	CM10 pH6	11768	0.00000079
6	CM10 pH4	6820	0.00000098
7	Q10 pH10	13144	0.00000156
8	Q10 pH10	13202	0.00000156
9	Q10 pH10	12656	0.00000185
10	CM10 pH4	5304	0.00000185
11	CM10 pH4	13185	0.00000185
12	CM10 pH4	5687	0.00000217
13	CM10 pH4	7245	0.00000217
14	CM10 pH6	12708	0.00000231
15	CM10 pH4	6892	0.00000253
16	CM10 pH4	5998	0.00000296
17	Q10 pH6	8868	0.00000459
18	CM10 pH6	6934	0.00000560
19	Q10 pH10	6358	0.00000600
20	CM10 pH6	7354	0.00000647
21	CM10 pH6	7463	0.00000647
22	Q10 pH10	15810	0.00000707
23	CM10 pH4	7836	0.00000850
24	CM10 pH6	10804	0.00000861
25	CM10 pH4	9843	0.00001140
26	Q10 pH6	7706	0.00001187
27	CM10 pH4	10612	0.00001318
28	Q10 pH6	8848	0.00001429
29	CM10 pH6	5409	0.00001507
30	Q10 pH6	7913	0.00002951
31	CM10 pH4	6257	0.00003088
32	CM10 pH4	10846	0.00003546
33	Q10 pH10	15510	0.00003955
34	Q10 pH10	7926	0.00004593
35	CM10 pH6	6863	0.00005050
36	Q10 pH6	7739	0.00005945
37	Q10 pH6	6747	0.00007055
38	CM10 pH6	10645	0.00007436

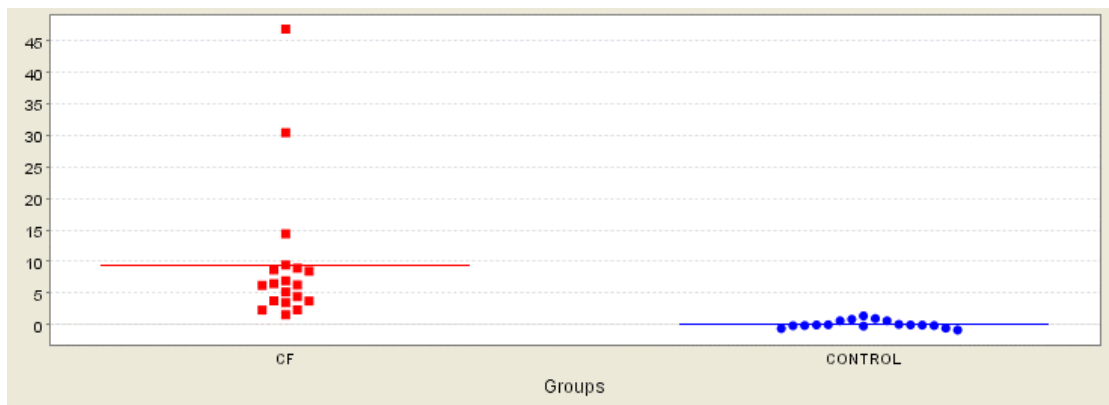
Table 16 - SELDI Induced Sputum Data – 38 potential biomarker

The 38 proteins listed above were detected in induced sputum.

1- CM10 pH 6 – 10589 Da, p=0.00000022



2- CM10 pH4 – 6340Da, p=0.00000031



3 - CM10 pH4-10187, p=0.00000031

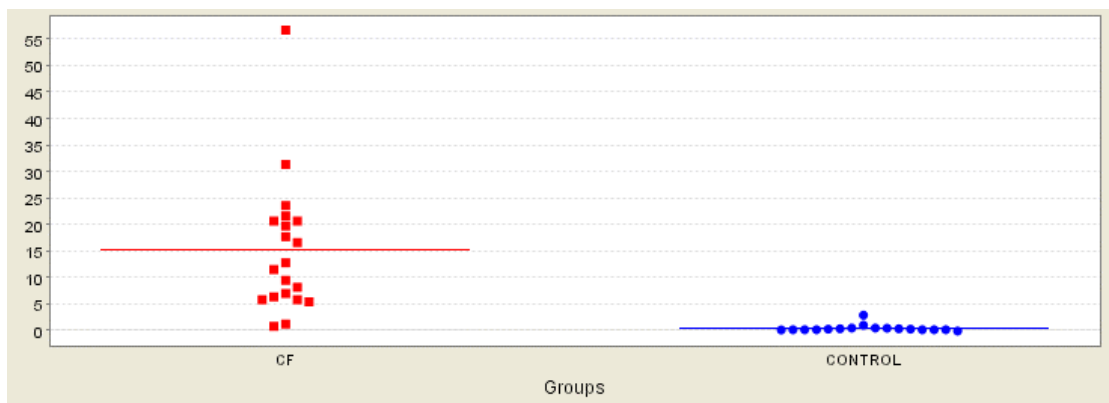
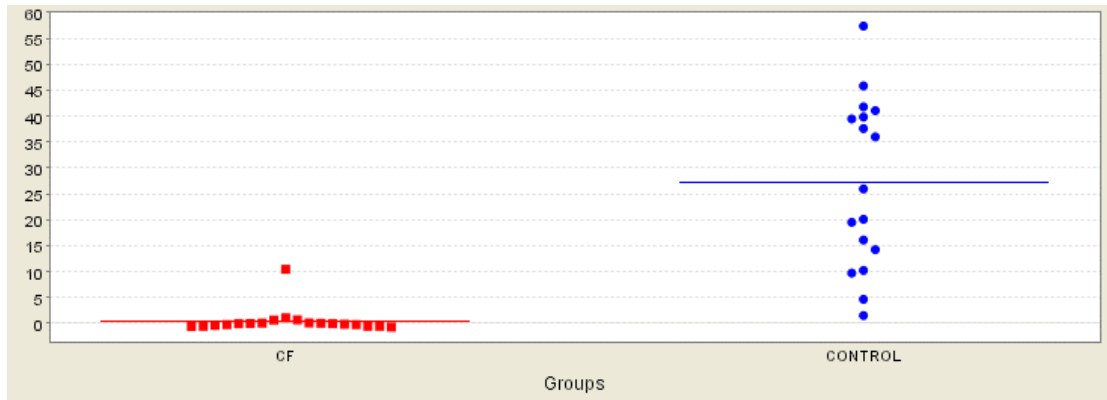
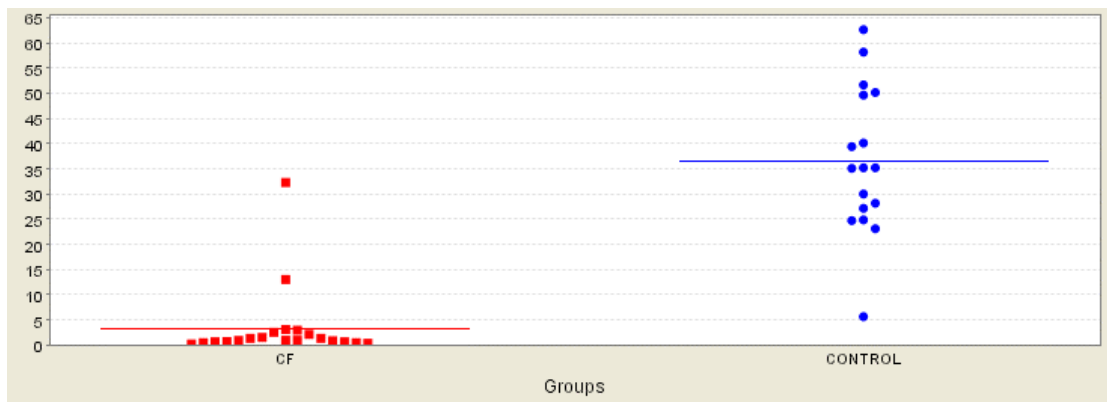


Figure 46. Induced Sputum - 6 examples (of 38) Biomarkers.

4 - CM10 pH4 – 5559 Da, p=0.00000060



5 - CM10 pH4 – 11768, p=0.00000079



6 – CM10 pH4 – 6820 Da, p=0.00000098

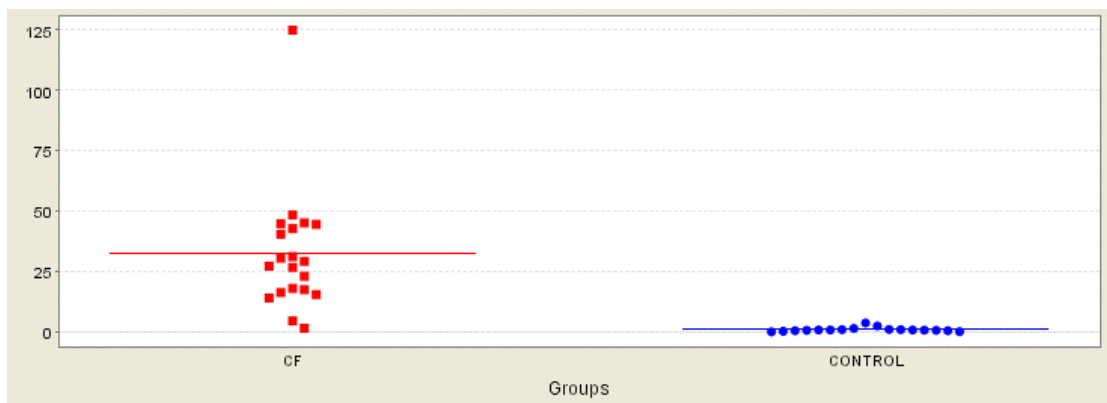


Figure 46. Induced Sputum - 6 examples (of 38) Biomarkers

6.6 Protein Identification

6.6.1 Introduction

Once the 38 biomarkers were detected in induced sputum using SELDI, the next step was to identify them. The technique employed in this thesis was to resolve the protein of interest onto a polyacrylamide gel and then to identify the protein using a combination of mass spectrometry and web based database searches.

The mass range of the 38 proteins identified in the induced sputum study was 5kDa to 13.5kDa. The first step was to separate the individual proteins in induced sputum samples. This was done using polyacrylamide gel separation. Induced sputum was taken from CF subjects whose sputum showed highest abundance of significant proteins.

6.6.1 Polyacrylamide Gel Electrophoresis

As the mass of proteins to be identified was relatively low (5-16kDa), the separating gels used needed to be of high density (i.e. 16%). A range of gels were tested with induced sputum and BAL and the gel which best resolution of proteins was the Invitrogen system and NuPAGE Novex Tricine Gel (16%) along with Novex Tricine running and sample buffers. These gels were marketed as giving good resolution of proteins in the 5 to 25kDa range. Gels were run using recommended protocols from Novex and Novex SimpleBlue SafeStain standards were used. Destaining of gels was performed with a rapid destaining technique using SimpleBlue SafeStain Microwave Protocol.

Once the protein of interest was visualised on the gel, it was punched out in pellets using a pipette tip. The gel was then destained and the protein eluted out of the pellets.

6.6.2 Trypsin Digestion

Gel pieces were covered with 100mM Ammonium Bicarbonate/50% Acetonitrile (50-100ul) and left for 15mins at room temperature. The supernatant was discarded and the procedure repeated three times until all stain was removed. Gel pieces were covered with 100% Acetonitrile for 10 mins. Again supernatant was removed and discarded and

gel “speed-vac” dried for 20 mins. Next 10mM DTT in 100mM ammonium bicarbonate was added to cover the gel pieces, and left at room temperature for 5 mins, before being incubated at 56°C for 1hr. The DTT was then removed by aspirating it with a pipette and 55mM Iodoacetamide in 100mM Ammonium was added. The gel was then incubated for 30 mins at room temperature in the dark, and the supernatant removed (this procedure was repeated 3 times). Gel pieces were dehydrated using 100% Acetonitrile for 10 mins, which was then evaporated using a vacuum extractor. Proteins were digested using Sigma modified Bovine Trypsin in 10% Acetonitrile, 25mM Ammonium Bicarbonate at 10-20ng/ul. Digestion occurred by incubating at 37°C for at least 16 hours.

6.6.4 Mass spectrometry techniques for protein identification

Once the protein of interest was selected out using gel electrophoresis and punched out, the punched out pellets were split into 2 portions. One portion was applied to a Normal Phase global binding surface SELDI chip and analysed to see if the correct protein was punched out and was relatively pure. The second portion was digested with trypsin overnight at 35°C.

Different mass spectrometry techniques were used once the protein of interest was selected out. The primary technique used was trypsin digestion and fragment identification using SELDI coupled to “Mascot” web searching. After this the peptide fragments were analysed by Quadrupole Time of Flight (Q-TOF) mass spectrometry for identification (this component was performed as a service by CIPHERGEN Biosystems (Copenhagen, Denmark). Independent identification was performed using a tandem mass spectrometry technique performed in collaboration with the University of Edinburgh proteomics facility - Edinburgh Protein Interaction Centre (EPIC).

6.6.5 Protein identification using SELDI

SELDI was used as the main technique to identify proteins from their peptide fragments. Once the trypsin digest of the proteins into peptide fragments was completed, the sample was put onto the SELDI chip surface. The resulting peptide fragment solution was then applied to Normal Phase global binding surface SELDI chip and analysed to see which peptide fragments were present. Confirmatory identification was performed using Quadrupole Time of Flight (Q-TOF) mass spectrometry.

6.6.6 Results

6.6.6.1 Protein 10589 - Identification using SELDI

The lead marker found in induced sputum was also one of the most abundant proteins in the induced sputum and BAL samples – at mass 10589. When digested, 8 peptide peaks were seen which were not attributable to trypsin. 6 of these 8 peptides had a mass within 1 dalton of predicted peptide fragments of Calgranulin A. This generated a Mowse Protein score of 105 (Mowse Protein scores greater than 62 are significant ($p < 0.05$)). 60% of the amino acid sequence for Calgranulin A was detected using SELDI (the fragments covered are displayed in bold). (Figure 47).

MLTELEKALN **SIIDVYHKYS** LIKGNFHAVY **RDDLKKLLET** CPQYIRKKG
ADVWFKELDI **NTDGAVNFQE** FLILVIKMGV AHKKSHEES HKE

Protein database searching was performed using the mascot proteomics database - http://www.matrixscience.com/search_form_select.html.

6.6.6.2 Identification using quadrupole TOF

Further to identification by peptide fragment mass, quadrupole TOF mass spectrometry was used (this was performed as a service by Ciphergen Biosystems, Copenhagen) to sequence two of the peptide fragments - 1272.69 and 1562.79. The two sequences were confirmed as ALNSIIDVYHK and GNFHAVYRDDLKK respectively and are both present in Calgranulin A. This gives confident identification based on the Mowse Ion Score which was 44 and 43 for each peptide respectively. A Mowse Ion score of greater than 38 indicates identity or extensive homology ($p < 0.05$).

6.6.6.3 Confirmatory Identification using tandem MS.

To confirm this finding – tandem mass spectrometry was used (this was performed in collaboration with Dr Andrew Cronshaw of Edinburgh Protein Interaction Centre (EPIC) at the University of Edinburgh).

Calgranulin A identification was confirmed and all 8 digested subunits were sequenced giving confident identification (figure 47).

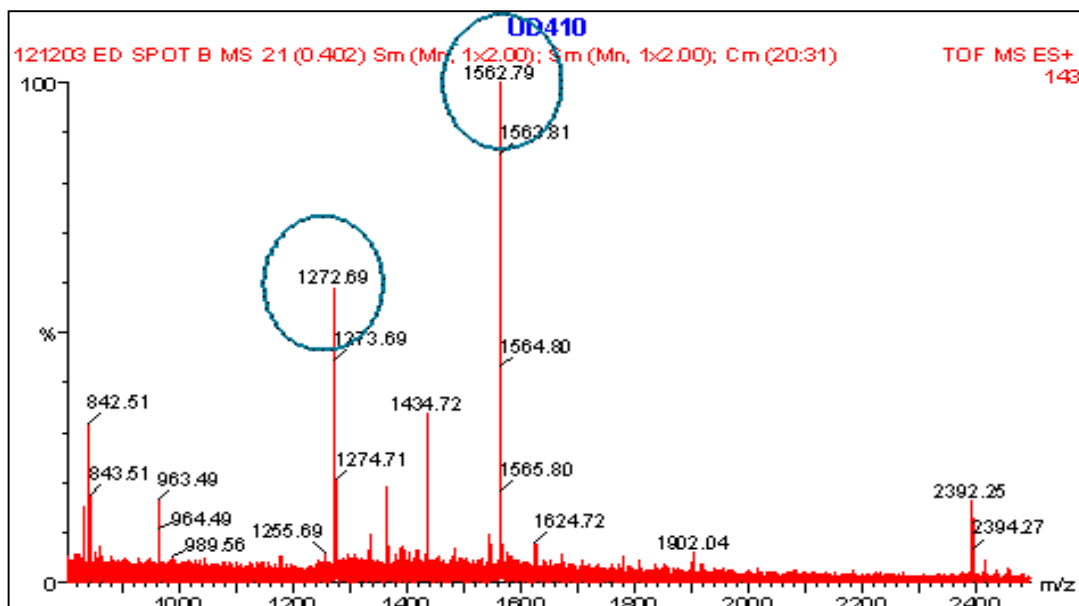


Figure 47. Peptide profile of protein 10589 digest (Calgranulin A) using quadrupole TOF.

The protein peptide fragments from digestion, by trypsin of the band at 10589Da.

Peptide fragments are seen from both the digested protein and from trypsin (autodigestion). 6 of the peptide masses matched Mascot database search for Calgranulin A. The 2 peptides selected for quadrupole TOF sequencing are circled in blue.

6.6.6.4 Protein 5165

The 5165 marker which showed complete discrimination in BAL fluid from children was upregulated in adults with CF but was not completely discriminatory. For identification purposes, it was very difficult to resolve onto a gel as it was of low abundance and had a relatively low mass. Once on the gel, the techniques outline above were applied and peptide digest using SELDI did not confidently identify any peptide fragments. Due to the low abundance of protein eluted from the gel, sequencing by either quadrupole or tandem MS was unsuccessful.

6.6.6.5 Other proteins

No other proteins were confidently identified. From the gel bands in the range 5-13.5kDa. Calgranulin B was detected using peptide homology. However attempts to confirm by sequencing was not successful. Haemoglobin alpha and beta chains, cytokeratin II were sequenced but did not have confident peptide matching.

Further work in the area of protein identification using 1D and 2D gel electrophoresis will continue, but is outwith the time restraints of this body of work.

6.6.6.6 Calgranulin A

The biomarker Calgranulin A has been identified with confidence and is known from the literature to be important in Cystic Fibrosis and in inflammation but has not been proposed as a biomarker of CF lung disease. It is also known as Cystic Fibrosis antigen (Wilson et al., 1977), S100-A8 protein, Migration inhibitory factor-related protein 8 or the Calprotectin L1L subunit .

Calgranulin has a predicted mass of 10828 Da. Using SELDI we assayed it at 10589.

The difference in mass is presumably due to post-translational modification e.g. cleavage from pro-protein. It is a member of the S100 protein family - characterised as low molecular mass acidic proteins and 2 calcium-binding domains .

It is expressed in neutrophils and infiltrating monocytes particularly during chronic inflammation. It is abundant in neutrophils and contributes 50% of the total cytosolic protein in resting neutrophils. Its exact function is not known but it is known to be a potent chemoattractant (Devery et al., 1994). It is secreted in response to inflammation and to bacterial LPS, and causes neutrophil recruitment from bone marrow (Vandal, Rouleau et al. 2003). Therefore, it is likely to be a marker of inflammation and neutrophil recruitment in CF lung disease.

Calgranulin has also been advocated as a biomarker of other diseases such as COPD (Merkel et al., 2005), transitional cell carcinoma of bladder (Tolson et al., 2005), ulcerative colitis (Tolson et al., 2005) and colorectal carcinoma (Hoff et al., 2004), and therefore cannot be considered to be specifically a marker of CF lung disease.

Previously, Calgranulin A had been reported as being a potential surrogate marker for CFTR function. It was noted to be raised in CF homozygotes as well as heterozygote

carriers (Wilson et al., 1977, Wilson et al., 1975). As CF heterozygotes do not have lung disease or any other signs of CF it may be postulated that calgranulin (previously termed CF antigen because of these data) has an activity related to CFTR protein rather than solely reporting on neutrophil activity (Hartz et al., 1998, van Heyningen et al., 1985). Calgranulin appears to be regulated by CFTR at a transcriptional level, in that if CFTR is absent, Calgranulin expression is upregulated (Renaud et al., 1994, Xu et al., 2003). Further investigation on the induced sputum of CF heterozygotes is warranted to clarify this.

Certainly a Calgranulin assay should be useful for future gene therapy trials, reporting on CF type inflammation and potentially on CFTR function. Further investigation of Calgranulin by SELDI and ELISA is warranted to investigate levels in subjects with CF, heterozygote carriers as well as control subjects with other diseases such as non-CF bronchiectasis.

It is encouraging that this lead biomarker found by SELDI shows such promise, and it is therefore important that efforts are made to identify the other lead biomarkers as well.

6.7 Proteomics discussion

The biomarkers discovered appear to be very sensitive at detecting CF lung disease. It may be that they are solely reporting on the inflammatory process rather than CFTR function. However markers found in the BAL and induced sputum were found to be different from non-CF inflammatory disease and healthy controls suggesting that some may be specific for CF lung disease and therefore may be directly reporting on CFTR dysfunction.

It is appropriate to pursue further validation of these markers longitudinally in CF subjects during periods of stability and during exacerbation and in relation to other inflammatory lung diseases, in particular, non-CF related bronchiectasis. Once identification of the proteins takes place, they have the potential to give new insights into CF pathophysiology.

These markers appear to be better discriminators than the gold standard non-invasive biomarker – FEV₁. As some show complete separation between groups, these offer high sensitivity and specificity. It is hoped that these markers would report on a small change in clinical status of the CF patient i.e. after 5 % transfection with wtCFTR gene therapy.

Using SELDI as an endpoint in gene therapy trials is likely to be cost effective in that it generates up to 38 different datapoints at around £15-20 per sample. However it is semiquantitative in nature and therefore specific ELISAs for each of the protein identified would have the advantage of being truly quantitative.

Until a CF gene therapy trial takes place, there is no real test of whether these markers will be sensitive enough to detect a change in patient status.

In the meantime, the best ways to validate these markers is to follow them up longitudinally in individual CF subjects during an infective exacerbation, and to test to see if they change when the patient improves from a stable baseline i.e. after a medical intervention such as nebulised antibiotic – Tobramycin (TOBI).

7.0 Final Discussion Chapter

7.0 Final Discussion Chapter

7.1 Hypothesis of thesis

Markers of inflammation in Cystic Fibrosis Lung Disease are measurable in samples collected non-invasively, and can be developed into clinically useful assays.

These assays would have the ability to reflect the level of

1. Inflammation in the CF lung.
2. CFTR function - this would be sensitive enough to detect improvement following administration of gene transfer agents

The hypothesis of this thesis can be broken down into two components, firstly that markers of inflammation can be measured in samples collected non-invasively and secondly that these markers could be developed into clinically useful assays, which reflect CF inflammation and CFTR function.

The first component, that markers can be detected in samples collected non-invasively has been shown to be true in both exhaled breath condensate and induced sputum.

7.2 Exhaled Breath Condensate

A large component of the work of this thesis was focussed on exhaled breath condensate because it was the most attractive sampling method i.e. it proved to be simple, safe, well tolerated and could be successfully performed in all subjects including young children. Such a sampling technique would have a positive impact on future gene therapy trials in that recruitment to a “non-invasive” group would be easier. It would allow samples to be collected in all subjects as well as allowing the study of subjects with poorer lung function (i.e. FEV₁ less than 40%).

Markers of inflammation were detectable in EBC. The majority of markers investigated were not discriminatory between CF and Non CF subjects. Two markers did emerge from the EBC research, pH and Ammonium. These markers did seem to reflect the inflammatory process as they are reduced in adults with cystic fibrosis. Therefore, EBC could be used as a sampling method for the non-invasive assessment of inflammation.

The process by which these markers are lower has not been established. The lower pH may be due to a reduction in activity of the three main proton pumps, generation of an inflammatory acid, or by a reduction in buffering capacity. The marker ammonium appears to reflect the proinflammatory cytokine activity that occurs in CF lung disease. Exhaled breath condensate therefore does meet the criteria that it can report on the increased airway inflammation in CF lung disease. The second component of the hypothesis that exhaled breath condensate can be used to assess CFTR function has not been proven. The ion concentrations in EBC were not different between CF and control subjects, in keeping with previous *vitro* and *in vivo* work. Therefore, they are unable to assess the chloride channel activity. From the point of view of bicarbonate secretion, it has not been possible to assay bicarbonate directly, and the fact that there is no difference in EBC pH between CF and Non CF children implies that EBC pH cannot be used to measure the postulated deficiency of bicarbonate in ASL. Therefore, EBC does not have the ability to reflect the level of CFTR function in subjects.

The measurement of EBC pH and ammonium as markers of inflammation should be used in future gene therapy trials as they are cheap, quick and simple to perform. It is noted that they do not completely discriminate between CF and non-CF subjects. This does not preclude their use in trials however, as the real power of such markers will be longitudinal data collection before, during and after gene therapy application. The strength of longitudinal data collection is that problems of intersubject variability can be avoided. The wide intersubject variability of EBC analysis perhaps reflects wide variability in ASL microdroplets collected. Longitudinal measurements in individuals with CF lung disease is even more important as CF exhibits variability in the day to day inflammatory state of the lung, combined with variability in the rate of disease progression. Therefore to achieve the highest possible power from gene therapy trials, the trial design which should be applied would be the gold standard double blind, placebo controlled, randomised trial.

7.3 Oxidative stress markers in Exhaled Breath and Exhaled Breath Condensate

The oxidative stress markers investigated in this thesis were exhaled carbon monoxide and the EBC marker 8-isoprostane. Both markers were unable to discriminate between

the stable CF subject and normal controls. As there is undoubtedly a marked inflammatory process evident clinically in the CF adult even when stable, these markers must therefore be relatively insensitive. Because of this relative insensitivity, it is unlikely that an improvement from the stable baseline would be detected. They should therefore not be used as endpoint assays of efficacy of gene transfer.

7.4 Nitrate stress markers in Exhaled Breath and Exhaled Breath Condensate

The nitrogen redox balance of the CF lung could be affected for three important reasons. Nitrate stress markers could be increased due to reaction of NO with reactive oxygen species in the neutrophil. The levels of NO that are available for this reaction are subject to change for two reasons. CFTR and iNOS levels appear to be co-expressed, and iNOS may be an interaction partner for CFTR, leading to reduced iNOS and reduced generation of NO in CF subjects. NO is also released from S-nitrosothiols (nitrated glutathione). As glutathione is reduced in the ASL of CF subjects, this mechanism may lead to reduced NO release. These two mechanisms for NO generation could potentially alter with successful gene therapy, causing an increase in NO expression.

The products of nitrate stress assayed were exhaled nitric oxide gas, nitrite and nitrotyrosine, which respectively are increasingly stable. There was no detectable difference between the stable CF subject and controls and therefore these assays were unable to discriminate the two groups.

However, it could be argued that NO and nitrite should be measured during gene therapy trials, as they are cheap simple non-invasive tests. The reasons they may change with gene therapy are due to possible induction of iNOS expression by WT CFTR and increased secretion of glutathione by functional CFTR.

7.5 Chemokines and Cytokines in EBC

Many markers were assessed with ELISA and with EIA techniques. The conclusion of my work is that appropriate standard curves need to be applied for EBC, and that with those investigated in this thesis there was no difference in chemokine or cytokine levels in EBC. Therefore, Chemokine and Cytokine assays of EBC are not suitable for use in gene therapy trials.

7.6 Proteomics and EBC

This thesis has shown that by using clean techniques, free from contamination, that there are no proteins in EBC that are repeatedly detectable using highly sensitive SELDI techniques. This technique reflects the highest sensitivity of any available proteomics instrument, due to its surface enhancement, and therefore until new technologies become available, it would be incorrect to assay any proteins in EBC.

7.7 Induced Sputum

The induced sputum data supported the findings of the BAL study, and identified 38 independent markers of CF lung inflammation. Therefore, sampling by collection of induced sputum should be used in gene therapy trials. The endpoints should be assessed by a combination of SELDI as an endpoint and by ELISA where this is available. The implications of this are significant in that non-invasive techniques could be used instead of BAL in future trials, obviating the need for general anaesthetics in children and restriction of recruitment of adults for trials to those who do not have respiratory failure.

The results show that there are proteins present in the BAL and induced sputum of CF subjects, which are different from healthy controls and subjects with other respiratory diseases. The markers are likely to be reporting on CF type inflammation but potentially could also be reporting specifically on CFTR function. Further studies in milder genotypes are warranted, as well as studies in subjects with non-CF bronchiectasis.

The marker Calgranulin is likely to report on neutrophil recruitment to the lung. It is hoped that this will be a sensitive marker of inflammation in the CF lung and it also has the potential to report on CFTR function due to calgranulins transcriptional association with CFTR, as it is raised in heterozygote carriers as well as homozygotes with CF.

Therefore, the non-invasive technique induced sputum coupled to proteomic analysis would have the ability to reflect the level of inflammation in CF subjects and may also report on CFTR function. It is hoped that these markers will be sensitive enough to detect successful wtCFTR gene transfer.

7.8 Concluding Remarks and Future Directions

The experiments described in this thesis have confirmed that non-invasive sampling of the ASL can provide biomarkers, which are likely to be sensitive and specific for CF lung disease. The principle that these biomarkers are likely to inform on the inflammatory status of subjects and potentially on CFTR function has been established. It remains to be seen whether they are sensitive and specific enough to report on successful CF gene therapy. In addition they may prove useful markers of inflammation in CF, helping to monitor patients longitudinally, and to help determine when patients might benefit from therapeutic interventions such as intravenous antibiotics.

The definitive validation of these markers can only come with successful gene transfer to the CF lung. CF GT trials are planned to start in 2009, and it is anticipated that the biomarkers identified in this thesis will be useful in identifying success of gene transfer. Until successful gene transfer occurs, we will not know how useful these markers will be. In the meantime, validation of these markers should take place through the longitudinal follow up of CF subjects through infective exacerbations and also after interventions which cause improvement from baseline – e.g. nebulised antibiotics.

7.9 Future directions

Further validation will continue, and as more sensitive proteomic and metabolomic tools are developed it is hoped that these will be applied to both EBC and Induced Sputum. The application of the techniques used in this thesis to other lung diseases such as Asthma, COPD and lung cancer may give further insight into the pathophysiology of such disease and could be used as tools to assist with the diagnosis and treatments of such diseases.

The full uses of the proteomic markers found in this thesis cannot be assessed until the markers have been sequenced and identified. Further identification should lead to assay developments such as ELISA which is quantitative, rather than semi quantitative (SELDI). Future work would include refinement of the analysis process, selection of the optimal surfaces for use in trials and identification of the causes of failed analysis i.e. samples which fail to “normalise”. It is hoped that at this stage proteomic

information from the ASL of CF subjects will inform on the pathophysiological processes in the lung. It should be considered that each of the biomarkers found in this thesis may not just report on the disease phenotype, but may be potential drug targets as causative agents in the inflammatory processes of CF lung disease.

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