Non-Invasive Biomarkers of Inflammation in the Assesment of Cystic Fibrosis Lung Disease

Robert Donald Gray

Submitted for the Degree of Doctor of Philosophy
The University of Edinburgh
2010

Table of Contents

Index of Figures

Index of Tables

Abstract

Cystic fibrosis (CF) is the most common fatal inherited single gene defect in Caucasian populations. CF lung disease is characterised by infection, inflammation and progressive lung destruction. Lung inflammation is measurable in CF patients even with early disease. Gene therapy offers a theoretical cure for CF lung disease, with large clinical trials now being planned. There is not only a clear need for the development of new therapies in CF but also the means to measure the success of these therapies. One possible approach is to measure biomarkers of airway inflammation non-invasively (in sputum or serum). In this thesis I have employed a number of techniques to measure potential biomarkers in sputum and blood in both cross sectional and serial samples following treatment.

Sputum was collected from patients with CF and a number of control groups including Asthma, Bronchiectasis, COPD and healthy controls. SELDI-TOF mass spectrometry was utilised to identify candidate protein biomarkers in sputum. Candidate biomarkers were then identified and compared to established biomarkers by ELISA in sputum. Emission spectroscopy was used to measure metal ions as non-protein biomarkers in sputum. SELDI TOF, ELISA and optical spectroscopy were used to measure biomarkers in CF sputum before and after exacerbation treatment. Calprotectin was also measured in serum before and after exacerbation.

SELDI TOF identified calprotectin as a marker of CF lung disease, which highly discriminated CF from control. This could also be measured by ELISA and compared favourably to other inflammatory markers such as Interleukin-8 (IL-8). Emission spectroscopy identified sputum zinc and iron as discriminatory markers of CF. Sputum calprotectin and zinc levels changed significantly following treatment of CF exacerbation. Serum calprotectin also changed significantly and could predict future outcome in these patients.

In this thesis I demonstrate the discovery and application of novel biomarkers of CF lung inflammation. I describe calprotectin (sputum and serum) as useful in the monitoring of exacerbation therapy, with similar findings being displayed for sputum zinc. Further work is now required to fully validate these findings for translation into clinically useful tools.

Declaration

This study represents original work carried out by the author, and has not been submitted in any form to any other University. Where use has been made of materials provided by others, due acknowledgement has been made.

Acknowledgements

I would like to thank Professor Andy Greening and Professor David Porteous for allowing me to conduct this research. I would also like to thank the Medical Research Council and Cystic Fibrosis Trust who funded this project.

I am extremely grateful to Margaret Imrie for her technical help and assistance throughout this project. I also thank Dr Chris Boyd and Dr Alastair Innes for their support and advice.

I would like to thank the staff of the Molecular Medicine Centre, University of Edinburgh; the staff and patients of the Cystic Fibrosis Clinic, Western General Hospital, Edinburgh; and the Staff of the Trace Element Reference Laboratory, Glasgow Royal Infirmary for their help and support.

Lastly I would like to thank my wife Katie and my Mum and Dad for love and support throughout this project.

1. Introduction

1.1 Background and Historical Perspective

Cystic Fibrosis is the most common fatal single gene disorder in Caucasian populations causing defects in the expression of the Cystic Fibrosis Transmembrane Regulator (CFTR), a chloride channel essential for the normal function of epithelial cells(1, 2). Defects in CFTR predispose the lung to bacterial infection and colonisation, repeated cycles of infection impacting on respiratory function. The median predicted survival of a patient born with CF in the United states in 2006 is 36.9 years (3), suggesting a vast improvement in patient care in recent years. In spite of survival improving over the past 4 decades progress has reached a plateau, and therefore CF patients are still more likely to die at a younger age than non-CF subjects. Respiratory decline is the cause of death 85% of cases (4), although other organs such as the pancreas and liver are often involved.

The term Cystic Fibrosis, although now widely used, is a relatively modern term, although descriptions of the disease are not new. German folklore from the middle ages dictates, *"woe to that child which when kissed on the forehead tastes salty. He is bewitched and soon must die"*(5). The first description *"Cystic Fibrosis"* as a clinical syndrome was made in 1938 by the American Pathologist Dorothy Andersen and described the particular (cystic) pancreatic pathology found in a group of neonates with high mortality(6). In 1949, it was first described as an autosomal recessive disease, but it was not until the mid 1980s that further advances were made into the understanding of the pathophysiology of CF with the characteristic

electrophysiology related to the impermiability of sweat ducts to chloride being described(7). This was followed by similar abnormalities in chloride transport also being observed in respiratory epithelium(8-11). It was not until 1989 however that the *CFTR* gene was finally identified and located to chromosome 7(12). Prior to this, several candidate genes and their products were proposed as the cause of CF including Calgranulin A (CF antigen)(13). Gene therapy was suggested as a potential curative treatment soon after the discovery of *CFTR* with the first such studies being published in the early 1990s(14, 15), although since these studies translation of gene therapy into a practical clinical treatment has been slower than initially anticipated.

1.2 The Basic Genetics of CF

CFTR is a large gene of around 250 kilobases located on the long arm of chromosome 7. In excess of 1000 candidate mutations have been identified and reported, the most common mutation being deletion of phenylalanine at position 508 (F508del), which occurs in 70% of northern European Caucasian CF patients. Other common mutations occur at frequencies of 1-3% and include R553X, G452X and 1717(G-A)(16). Although genotype aids in the diagnosis of the patient, the prediction of phenotype is less reliable, particularly in reference to pulmonary disease(17). Indeed the development of the pulmonary complications of CF is widely variable and as such it is not uncommon for patients to be diagnosed with CF in adulthood(18), particularly if associated with less common mutations.

1.2.1 Molecular Classification of CF Genetic Mutations

In order to understand the pathophysiology of CF, mutations in CFTR have been classified in terms of the molecular dysfunction of the chloride channel including failure of production, processing or transport of the protein to the apical membrane of epithelial cells. Five main types of mutation have been described.

1. Class I mutations. These are due to premature stop mutations in the CFTR sequence resulting in termination of translation and protein synthesis. Examples include G542X. These mutations are rare and account for only 7% of CF subjects(19).
2. Class II mutations. These lead to the production of defective CFTR that cannot be properly trafficked to the apical cell membrane(20), such as F508del which affects up to 70% of CF patients.
3. Class III mutations. These lead to the production of dysfunctional CFTR which is trafficked to the cell membrane but is not properly regulated(21). Common mutations include G551D accounting for around 3% of CF patients.
4. Class IV mutations. These lead to CFTR that has abnormal ion conductance and thus is dysfunctional(22). Common mutations include R117H.
5. Class V mutations. These mutations include splice mutations leading to the complete or partial production of the CFTR protein and reduced protein synthesis(23). Examples include A445E.

Although this classification is useful as a guide to the molecular defect the prediction of clinical phenotype is less easy and therefore clinical classifications of CF mutations have also been used to describe CFTR function.

1.2.2 Clinical Classification of CF Mutations

CF genetic mutations have been classified as severe or mild with respect to exocrine pancreatic function. Patients homozygous for the F508del mutation frequently display severe pancreatic insufficiency(24). Those homozygous for the F508del mutations have higher chloride levels on sweat testing suggesting greater chloride channel dysfunction, and male homozygous F508del patients would be expected to be infertile due to obstructive azoospermia(25). The relationship of genotype to lung function is less clear, and even those with F508del mutations display a wide range of disease from mild to severe. Furthermore genotype does not predict chloride transport as measured by nasal potential difference(26), a common measurement used in clinical trials of new treatments, and thus suggests a subtly different effect of F508del mutations in the sweat gland and respiratory mucosa.

The reason for the apparent mismatch between CF genotype and severity of lung disease is presently unclear although other genes, so called "modifiers", may be involved. For example, polymorphisms of the transforming growth factor beta gene may be associated with increased severity of lung disease(27) and conversely mutations in the enhancer region of the alpha one antitrypsin gene may lead to better prognosis in lung disease(28). Therefore although a monogenic disease by definition, the clinical syndrome of CF depends on the complicated interplay of a number of genes, which ultimately, may interact to characterise the disease for any given individual.

1.3 The Cell Biology of CF

1.3.1 CFTR

CFTR shares homology with a family of proteins known as ATP-binding cassette (ABC) transporters(12), which function as pumps that export macromolecules from the cell interior to exterior utilising ATP(29). CFTR has 5 proposed domains including 2 transmembrane domains spanning the lipid bilayer: two nucleotide binding domains (NBD) and a cytoplasmic regulatory domain(12). An adapted illustration of this is displayed in figure1(30). CFTR is found in epithelial cells lining the lung, gut, pancreas and liver among other organs. The dysfunction, presence of mutant, or absence of this protein channel leads to the clinical syndrome of CF.

As well as having function as a chloride channel CFTR also has a key role in the regulation of other ion channels. CFTR has also been implicated as a bicarbonate transporter and an ATP transporter as well as being suggested as a receptor for *Pseudomonas aeruginosa.*

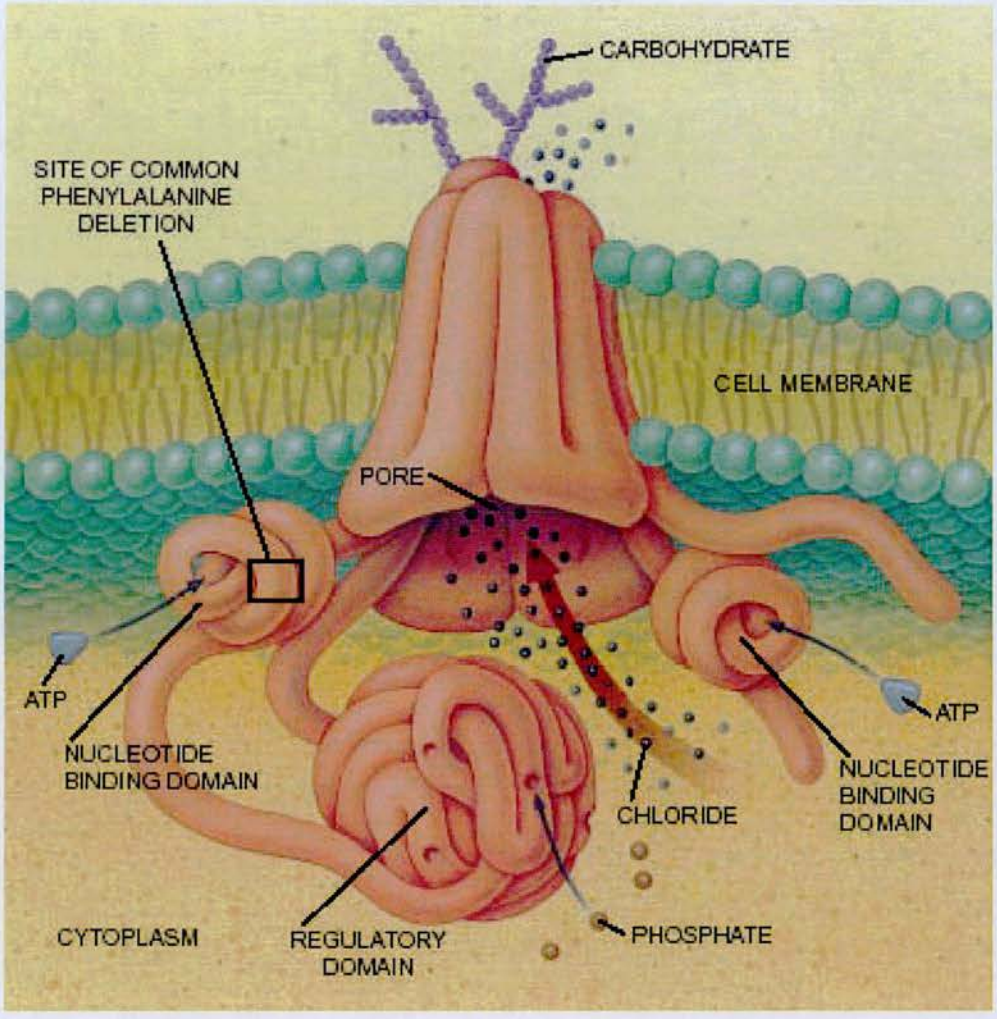


FIGURE 1.1 PROPOSED STRUCTURE OF CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) PROTEIN

CFTR is a chloride channel situated at the apical membrane of epithelial cells. CF mutations lead to abnormal protein function or expression and as such abnormalities in chloride transport. The site of the common F508del mutation is demonstrated in the Nucleotide Binding Domain(30).

1.3.2 Normal Function of CFTR

The activity of CFTR as a chloride transporter was demonstrated by showing defective chloride transport in CF airway epithelial cells, characterised by an inability to respond to cyclic adenosine monophosphate dependent protein kinase, which is an inducer of chloride transport in normal cells(11, 31, 32). Conversely if CFTR is expressed in cells that do not normally display endogenous CFTR, such as xenopus oocytes, then chloride conductance can be induced via the cAMP-dependant protein kinase pathway(33-35). Furthermore, expression of wild type CFTR in CF epithelial cells leads to the restoration of chloride transport(36, 37). Thus normal activity of CFTR is central to regular chloride transport. Confirmation of CFTR as a chloride channel and not just a regulator of chloride transport was demonstrated by altering chloride permeability in cells following site directed mutagenesis of *CFTR* to create mutants, and then the incorporation of CFTR into the lipid bilayers of these cells to form chloride channels and allow chloride transport similar to wild type epithelial cells(38, 39).

The regulation of CFTR (opening and closing) is proposed to occur through cycles of phosphorylation and dephosphorylation in conjunction with ATP hydrolysis(40-43). The activation of cAMP dependant protein kinases cause phosphorylation of multiple serine residues in the regulatory domain with subsequent hydrolysis of ATP at the nuclear binding domain causing the channel to open and allow the free flow of chloride ions.

As well as functioning as a chloride channel CFTR may also act as a regulator of other ion channels such as the amiloride sensitive epithelial sodium channel (ENaC),

suggested by abnormally elevated sodium absorption in CF airway cells compared to control(44), which is again corrected by transfecting CFTR in similar cells(45). Furthermore co-expression of *CFTR* and *ENaC* in constitutively non-expressing cell lines leads to lower basal sodium currents suggesting direct inhibition of ENaC by CFTR(46). This inhibition is thought to be secondary to effects on channel function rather than channel number(47, 48). The loss of regulation of ENaC in CF can therefore explain the findings of low chloride secretion and increased sodium absorption thought to be important in CF lung disease (see later). This relationship was further confirmed with an overexpressing ENaC transgenic mouse which has been shown to develop a lung phenotype similar to that seen in CF(49) suggesting that the interaction of CFTR with ENaC has a major pathogenic role in CF lung disease.

CFTR may also have effects on other ion channels such as the outwardly-rectifying chloride channel(50); calcium dependant chloride secretion(51); bicarbonate transport via chloride coupled bicarbonate exchanger(52) and potassium transport via the renal potassium channel ROMK2(53), although the significance of these interactions is unclear.

A further possible function of CFTR is as a receptor for *P. aeruginosa,* by which epithelial cells can bind and internalise bacteria as a mechanism of clearance(54, 55). CF epithelial cell lines are less able to internalise *P. aeruginosa* than wild type cells and this has been suggested by some researchers as a crucial defect in CF pathogenesis, allowing the colonisation of the lung with bacteria and subsequent

inflammation and damage (although it should be considered that the majority of established early infections in CF are with gram positive organisms(3)). The binding of *P. aeruginosa* is proposed to take place between the outer core bacterial lipopolysaccharide and the first extracellular domain of CFTR. Recently it has been suggested that the binding of *P. aeruginosa* to CFTR initiates an inflammatory response that is crucial to the removal of bacteria(56). The interaction of *P. aeruginosa* with CFTR may therefore be implicated in the pathogenesis of lung disease, although evidence from animal models of CF and clinical samples will be required to confirm this.

1.4 Multi-System Problems in CF

CF can affect any organ system in the body that has an epithelial lining. Thus the skin (in particular the sweat glands), reproductive tract, bowel, pancreas and the liver may be affected(1, 2) as well as the lung.

1.4.1 Skin

In the skin CF leads to the secretion of salt rich sweat with the dysfunction of CFTR not allowing normal chloride ion resorption, thus leading to the accumulation of high levels of salt in the sweat glands (57-60). This defect has been exploited clinically as the sweat test, a commonly used to diagnostic test for CF. Furthermore CF can lead to salt depletion and dehydration in small children(61), a phenomenon first observed during a heat wave in New York in the 1950s(62).

1.4.2 Gastrointestinal Disease

The majority of patients with CF suffer from pancreatic disease(2), indeed the nomenclature cystic fibrosis is based on the pathological findings in the pancreas of patients at post mortem(6). In the pancreas CFTR normally exchanges chloride ions in exocrine secretions for bicarbonate to neutralise and inactivate pancreatic enzymes. In the absence of CFTR, pancreatic enzymes are constantly activated with the end result auto digestion and destruction of the pancreas. This is further compounded by protein hypersectretion in the pancreas, protein plugging, and relative luminal dehydration causing ductal blockage(63). Low levels of bicarbonate in secretions also impair gastric acid neutralisation in the duodenum, leading to the failure of digestive enzymes and malabsorption. As well as developing exocrine pancreatic insufficiency, the chronic inflammation of the pancreas may lead to islet of Langerhan atrophy and consequently insulin dependant diabetes mellitus(64), which is seen in many adult CF patients.

Intestinal disease may also be a complicating feature of CF. Meconium ileus (obstruction of the small bowel by thick mucus) is observed in 10 -20% of new-borns with CF(65). In older patients distal intestinal obstruction syndrome due to thickened mucous and incomplete digestion of food is experienced by up to 20% of patients. A finding replicated by many mouse models of CF which have severe gastrointestinal pathology(66).

Hepatobiliary disease is a further potential complication of CF below the diaphragm. Dysfunctional secretion of chloride and bicarbonate into the biliary tract leads to concentrated and acidic bile, but interestingly the majority of bile duct disease is

intra-hepatic rather than effecting the common bile duct and gall bladder(67), and thus complications include cirrhosis and portal hypertension. In a recent case series from a large UK CF centre 154 patients with abnormal liver function and CF were followed or 5 years(68), and although 29 of these patients developed portal hypertension only 1 patient developed liver failure requiring transplant.

1.4.3 Problems with Fertility

Infertility is seen in almost all male patients due to obstructive azoospermia with congenital bilateral absence of the Vas Deferens (CBAVD) being common. Fertility is maintained in many females (69), and if affected this is most likely secondary to abnormalities in the menstrual cycle caused by chronic ill health and poor nutrition related to underlying respiratory disease rather than CF mutations per se. Interestingly CBAVD as well as being seen in virtually all male CF subjects is also recorded in the general population, with CFTR function of at least 10% thought to be necessary for normal embryological development of the Vas Deferens. Indeed CBAVD in non-CF patients is associated with a higher incidence of carrying a CF mutation(25), than the general population. In spite of this, advances in fertility treatment have allowed adults with CF (even males) to have children.

1.5 CF Lung Disease

Decreased CFTR function is most significant in terms of mortality and morbidity in the lung, with 85% CF deaths being due to the complications of pulmonary disease(4). Many of the present therapies for CF are thus focussed at attempting to correct or treat the pulmonary complications of CF.

1.5.1 The Development of CF Lung Disease

The lungs of most CF patients are histologically normal at birth aside minor morphological changes in the sub-mucosal glands(70-72), but during life there is a gradual progression of disease throughout the respiratory tract leading to small airway damage but mostly sparing the alveolar space(73), possibly reflecting the low levels of CFTR in that area. In spite of the absence of frank histological changes at birth, some studies have demonstrated the presence of increased levels of airways inflammation with both interleukin-8 and neutrophil elastase being found in increased levels in children with CF at 6 months of age(74). This is consistent with the measurement of raised inflammatory mediators when CFTR is knocked out in rats in-utero(75), and the increased levels of neutrophil proteins described in some mouse models of CF(76). The reason why dysfunction of CFTR leads to inflammatory lung disease are poorly understood. However it is widely accepted that the basic defect in CF leads to changes in the airways surface liquid (ASL), the fluid in the airway that bathes the cilia facilitating normal mucociliary clearance, and thus the removal of harmful stimuli such as bacteria and inflammatory proteins is impaired.

The ASL is a thin film of fluid (~30 μl) covering the airway surface in which foreign inhaled matter is trapped and then moved by beating cilia towards the larynx for disposal by expectoration or swallowing. Decreased mucociliary clearance is well described in CF, although measurement is impaired by variability in the variety of techniques used(77). As well as allowing free flow from the lung the ASL contains a number of molecules that are central to the innate immune response to bacterial infection in the lung. Antimicrobial proteins such as lactoferrin, secretory leukocyte

protease inhibitor (SLPI), surfactant proteins A and D, peroxidises, cathelicidins and beta defensins among many molecules are present in ASL. Therefore any alteration in the physical properties of ASL may lead to both abnormal clearance from the lung as well as innate immune dysfunction. The effects of CFTR dysfunction on ASL have thus led to a number of different hypotheses.

1.5.2 High Salt vs. Low Volume Hypothesis

Two main hypotheses have been developed to explain the effects of CFTR dysfunction on ASL: the high salt hypothesis and the low volume hypothesis. The high salt hypothesis suggests that normal ASL is hypotonic with a low salt concentration, and this environment is essential for the ability of innate immune molecules, in particular beta defensins to kill bacteria(78, 79). This hypothesis assumes that, as with the CF sweat gland, reduced chloride resorption by epithelial cells leads to decreased sodium absorption and thus higher than normal salt levels in ASL. This was supported by the apparent measurement of increased levels of sodium chloride in CF ASL(80). This is not a consistent finding in all studies(81), and has led to other explanations for the effects of CFTR dysfunction on the airway, most prominently as the low volume hypothesis(82).

The low volume hypothesis suggests that ASL has salt concentrations similar to plasma levels (i.e. normal) and that with CFTR dysfunction the activity of ENaC, the inducible sodium channel, is increased leading to over absorption of sodium and thus water from the airways surface, causing dehydration and a loss of ASL volume which interferes with normal mucociliary clearance(83). This hypothesis relies on

there also being chloride reabsorption via paracellular pathways and calcium-activated chloride channels. As well as decreasing mucociliary clearance, airways dehydration increases the viscosity of mucus, thus creating a more favourable environment for bacterial colonisation and infection.

Several studies have since demonstrated no difference in ASL tonicity between CF and non-CF in man and mouse ASL thus supporting the low volume hypothesis(81, 84-86). Furthermore a transgenic mouse over-expressing ENaC develops a pulmonary disease phenotype similar to that of CF due to depletion of the airways surface liquid volume(49), thus underlining the potential role for ENaC and airways dehydration in CF lung disease.

The overriding evidence for the detrimental effects of CFTR dysfunction in the development of CF lung disease is that with a decrease in airways surface liquid there is impairment of mucociliary clearance increasing the susceptibility to infection and the damaging effects of lung inflammation. This suggests an alternative function for CFTR in the airway and the sweat gland. In particular with under absorption of chloride in the sweat gland due to CFTR dysfunction; but removal of ENaC inhibition in the lung leading to a decrease in mucociliary clearance following sodium hyperabsorption.

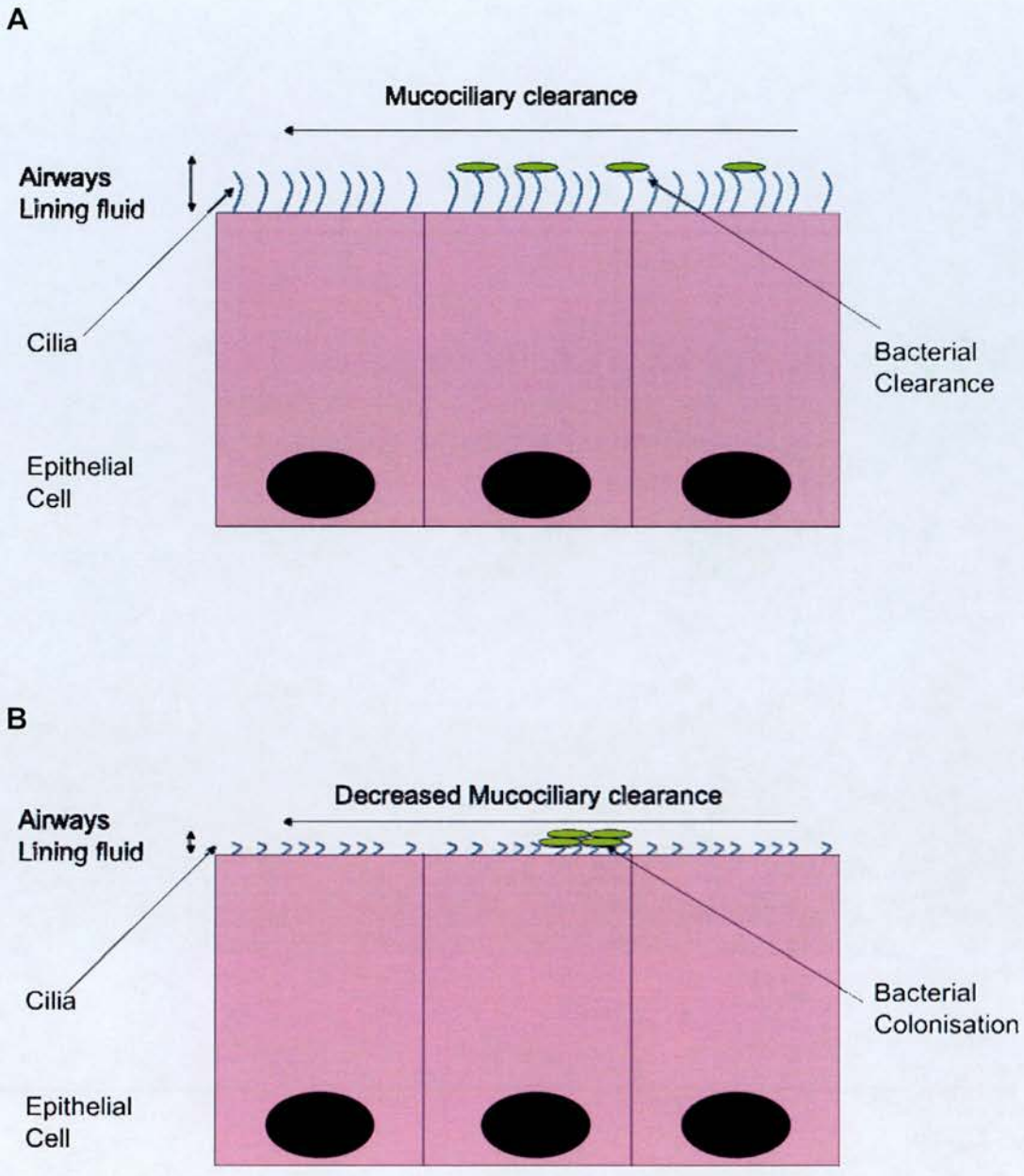


FIGURE 1.2. THE LOW VOLUME HYPOTHESIS.
A. Normal airway surface liquid properties allow efficient mucociliary clearance of bacteria (and inflammatory cells/material) from the small airways to the trachea, which is then cleared, by coughing and expectorating or swallowing. B. The increased absorption of sodium and water from the airway leads to depletion of the ASL, therefore decreasing mucociliary clearance and allowing bacterial colonisation, subsequent infection and lung damage by inflammatory mediators.

1.5.3 Lung Inflammation

Inflammation may be detected in the CF airway even in the absence of proven infection(74). The inflammatory response to infection is more pronounced in CF with a more intense and longer lasting inflammatory response being observed following infection with lower respiratory pathogens(87). It is commonly held that in CF infection leads to chronic inflammation and consequently lung damage creating a vicious cycle of lung infection, inflammation and damage(88). However lung inflammation has been detected in some studies prior to infection(74, 89) and therefore this area remains controversial. Nevertheless, regardless of the temporal association the CF lung is prone to respiratory infection and inflammation leading to damage as a consequence. A further aspect of CF lung inflammation is the dysregulation of inflammatory cytokines, with increased levels of pro-inflammatory mediators such as interleukin (IL)-8 and decreased levels of anti-inflammatory cytokines such as IL-10 being observed in the CF airway compared to control populations. Thus the interplay between infection and inflammation may be further confounded by dysregulation of the normal inflammatory response.

The major effector cell in the (central) CF airway is the neutrophil, which is able to secrete proteoloytic enzymes such as cathepsin G and neutrophil elastase (NE). Increased NE activity is readily measureable in the CF airway and these and other proteases may overwhelm the natural anti-protease system in the lung, contributing to destruction of lung tissue (52) and cleaving opsonins, thus decreasing effective bacterial phagocytosis and increasing IL8 production. Neutrophils are normally cleared from sites of acute inflammation by apoptosis and phagocytosis(90), however in the CF airway a greater number of secondary necrotic neutrophils are present

suggesting a decrease in clearance of apoptotic cells(91). Furthermore neutrophil elastase may delay the clearance of apoptotic cells by cleaving phosphatidylserine receptors on macrophages decreasing the recognition and phagacytosis of apoptotic neutrophils(92). Secondary necrosis of neutrophils may then lead to the release of pro-inflammatory mediators, further driving inflammation in the CF lung and as such the products of secondary necrosis may provide a suitable substrate to discover biomarkers of disease. Other forms of neutrophil cell death such as the recently described phenomenon of neutrophil extracellular trap (NET) formation may also be important in the release of neutrophil products such as neutrophil elastase and myeloperoxidase(93), although further work will be required to elucidate a role for NETs in CF.

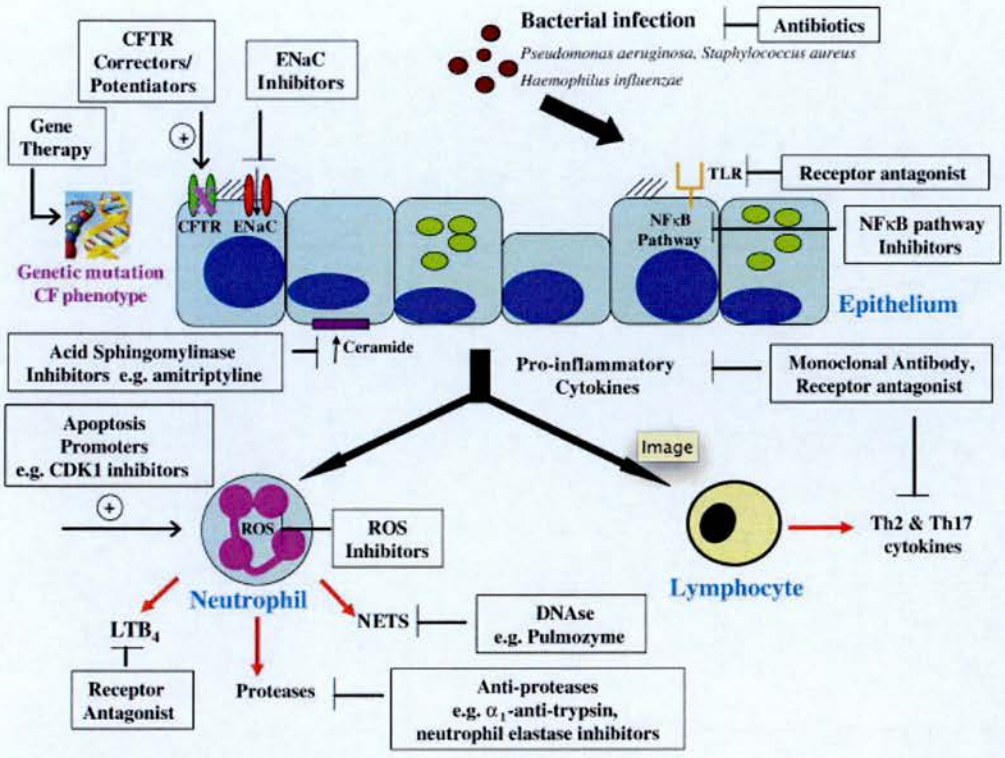


FIGURE 1.3 TARGETS FOR TREATMENT OF INFLAMMATION IN CYSTIC FIBROSIS

This figure demonstrates a number of potential targets for the treatment of Cystic Fibrosis including gene therapy to correct the CF gene mutation, potentiation of low level CFTR dysfunction or even modulating the inflammation seen as a consequence to CF mutations. Further strategies also include the modulation of inflammatory cells such as neutrophils and lymphocytes in the CF lung and monoclonal antibodies to block the activity of key cytokines (adapted from workshop of targets for inflammatory treatment)(93).

1.5.4 Lung infection in CF

Chronic lung infection is a hallmark of established CF lung disease with *Staphlococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* being the most common pathogens, with a prevalence of 52%, 17% and 55% respectively in the CF Foundation annual registry report of 2006 (3). In infants with CF the most common organisms cultured are *S. aureus* and *H. influenzae*. Following initial colonisation the airway is likely to be more prone to further infection with organisms such as *P. aeruginosa*. Whether this is a direct priming effect of the early infection or the result of early lung damage following infection is unclear at present. Intermittent *P. aeruginosa* colonisation followed by permanent infection is often observed, suggesting that even in patients with CF that the initial clearance of this organism may be possible. However in CF *P. aeruginosa* which is normally present as a motile non-mucoid phenotype, can transform into a non-motile mucoid phenotype at this time, allowing permanent infection of the lung. The presence of mucoid phenotype *P. aeruginosa* in CF is associated with deterioration in lung function(94, 95), and therefore patients with non-mucoid forms of *P. aeruginosa* have better lung function than those infected with mucoid strains(96). Once established in the lung *P. aeruginosa* becomes increasingly difficult to treat due to its ability to mutate, form alginate and develop biofilms(97), thus rendering it less susceptible to penetration by antibiotics. As CF lung disease progresses other organisms such as *Burkolderia cepacia complex* and *Stenotrophomonas maltophilia* are also implicated as pathogens as a consequence of the increased susceptibility of the damaged lung to infection thus further complicating the lung environment in CF patients. Indeed by adulthood many patients are chronically infected with multiple pathogens, making treatment with antibiotic therapy increasingly difficult.

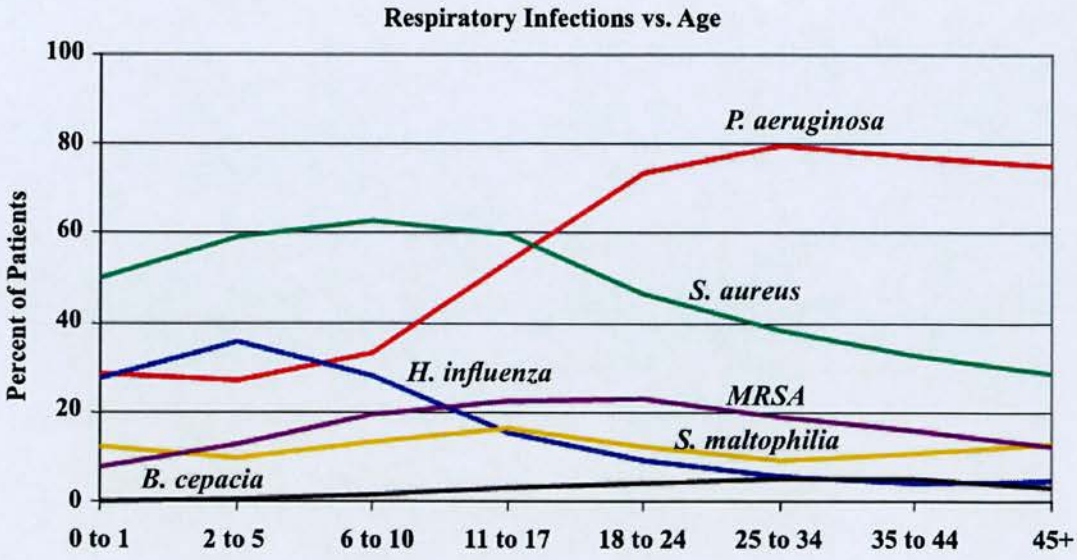


FIGURE 1.4. CF LUNG INFECTION BY PATHOGEN

CF lung disease is characterised by infection. The most common organisms are demonstrated in this figure and include *Staphylococcus aureus, Haemophilus influenza* and *Pseudomonas aeruginosa*. The incidence of *P. aeruginosa* infection increases with age and becomes the dominant pathogen by adulthood. This figure is adapted from the CF Foundation Patient Registry 2006 report(3).

1.5.5 CF Lung Disease Exacerbation

As well as having chronic bacterial colonisation patients with CF also experience recurrent episodes of increasing pulmonary symptoms termed exacerbations which are accompanied by a decrease in lung function(98). The aetiology of these episodes is unclear but the acquisition of new pathogenic organisms(99) or an increase in density of colonising organisms(100) have both been suggested as possible aetiological factors. Viruses such as respiratory syncitial virus (RSV) have been implicated as important initiating factors of pulmonary exacerbation in children(101) Certainly, treatment of exacerbations with antibiotic therapy decreases the bacterial density of respiratory secretions(102, 103). And both the treatment of bacterial colonisation with nebulised anti-pseudomonal treatment(104) and nebulised therapy to decrease the viscosity of respiratory secretions(105) decrease the need for exacerbation treatment as well as improving lung function. Exacerbations represent a complicated imbalance of multiple factors including bacterial burden, host defence in combination with physical factors such as increases in mucus viscosity. Nevertheless exacerbation represents an important part of the pathophysiology of established CF lung disease, and may also allow the study of a changing state of inflammation and infection in CF patients.

1.6 Present Therapy for Management of CF lung Disease

Mortality and morbidity in CF lung disease have improved steadily over the past 30 years, and as such a once universally fatal disease of childhood can now be managed effectively to allow patients to live into adulthood. Nevertheless the predicted median survival of around 36 years will only improve significantly if therapy is

focused on the correction of the underlying molecular defect in CF rather than it's consequences. The present treatment strategies, which aim to control the consequences of CF lung disease such as infection and inflammation, are summarised below.

1.6.1 Aerosolised Antibiotics

In view of the highly pathogenic nature of *P. aeruginosa* infection, aerosolised antibiotics have been advocated for the early eradication of PA infection as well as the suppression of chronic infection. Studies using nebulised tobramycin have demonstrated significant improvements in lung function when compared to placebo and standard therapy (104, 106, 107), with fewer exacerbations and less time spent in hospital, and also improvements quality of life (104). This is further supported by Cochrane review(108). Therefore aerosolised anti-*pseudomonal* antibiotics have been recommended for all patients 6 years or older with persistent PA infection with moderate to severe lung disease(109).

1.6.2 Nebulised Recombinant DNAse

Cystic fibrosis sputum contains large amounts of cellular debris including DNA, which increases the viscosity of mucus due to electrostatic interactions. The use of DNAse as a strategy for decreasing the viscosity of CF secretions was first described in the 1960s when bovine DNAse was utilised, these trials were however limited due to the immunogenicity of the bovine product. More recently recombinant DNAse (dornase alfa) was developed as a strategy to degrade DNA, decreasing the viscosity of mucus and thus facilitating mucociliary clearance. Several multi-centre trials have

assessed the long-term efficacy of DNAse on lung function in patients with moderate to severe disease(105, 110). Furthermore Fuchs and colleagues demonstrated an improvement in incidence and length of pulmonary exacerbation in DNAse treated patients(105), and its use is supported by Cochrane review(111). It has also been demonstrated that in mild disease nebulised DNAse also reduces number of exacerbations and maintains lung function(112). As such the use of DNAse is recommended in the treatment of moderate to severe patients over 6 years and may even be indicated in the treatment milder lung disease(109).

1.6.3 Hypertonic Saline

Another strategy to increase mucociliary clearance in CF is by osmotically hydrating the airways surface liquid by the use of hypertonic saline (HS)(113). HS both improves lung function(114) and decreases the number of pulmonary exacerbations(115), which is remarkable for such a simple therapy. Furthermore HS is well tolerated by patients with few adverse events being recorded in published studies, leading to the study of further osmotic agents in the airway (116). Therefore the use of HS may be indicated all patients over six years to improve lung function and exacerbation frequency(109).

1.6.4 Anti-Inflammatory Medications

As excessive airways inflammation is a hallmark of CF lung disease, treatment with various anti-inflammatory agents have long been proposed as possible management strategies. Inhaled corticosteroids (ICS) however, the mainstay of treatment in asthma (an allergic inflammatory disease), have recently been demonstrated

ineffective in CF in a study that followed ICS withdrawal in a large number of patients(117). A large multi-centre trial carried out in a children demonstrated an improvement in lung function in subjects taking oral corticosteroids(118), however a Cochrane review suggested that the side effects related to long term oral corticosteroid outweigh any benefit in lung function improvement(119). As such corticosteroid therapy is not recommended in CF unless it is used to treat concomitant allergic airways disease(109).

Treatment with non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen have proved a successful strategy in terms of slowing the decline in lung function in CF over a 4 year period in patients with mild lung disease(120), although the mechanism of anti-inflammatory activity in the CF airway is not well described. In view of these findings high dose ibuprofen is recommended as treatment to slow the loss of lung function in patients with CF over 6 years old with mild disease (FEV_1 >60% predicted)(109). This treatment must however be used with caution due to the potential side effects of NSAID treatment such as gastro-duodenal ulceration and renal impairment. Other anti-inflammatory drugs such as leukotriene receptor antagonists and sodium cromoglycate are not presently recommended in CF due to a lack of demonstrated treatment effect(109).

1.6.5 Macrolide Therapy

In view of the positive benefits of macrolide antibiotic treatment in the lung condition diffuse Japanese pan-bronchiolitis (121) (a disease characterised by *P. aerugenosa* colonisation), macrolides were suggested as a possible treatment agent in

CF with its proposed anti-microbial and anti-inflammatory activity. Several studies using the macrolide azithromycin have demonstrated a significant improvement in lung function(122-124) in CF patients. In those patients chronically infected with *P. aeruginosa* there was a significant decrease in exacerbation frequency(122) and therefore long term azithromycin treatment is recommended in all CF patient six years and older with persistent *P. aerugenosa* colonisation(109).

1.6.6 Treatment of CF Lung Exacerbation

No unifying diagnostic criteria have been developed for the diagnosis and treatment of CF exacerbation and indeed differences in criteria exist between those treating adults and children(125). In spite of no new organism being cultured at the time of exacerbation in most subjects antibiotic therapy remains the mainstay of treatment(126). In general, combinations of antibiotics rather than monotherapy are used due to the concern of bacterial resistance to antibiotics developing(127), although this is not supported by a recent Cochrane review(128). Combination antibiotic therapy is usually in the form of two antibiotics with differing modes of activity. Interestingly a recent study demonstrated no benefit in testing the synergistic combination of antibiotics in the laboratory compared to physician best choice in terms of patient outcome(129). Although antibiotics form the mainstay of therapy, physiotherapy and nutritional support are also of importance(126), and there is no evidence as of yet that anti-inflammatory treatment is indicated in CF exacerbation.

1.6.7 Lung Transplant

Lung transplant offers the only theoretical "cure" for CF lung disease although this is not without limitations. Strict criteria exist for lung transplantation and not all patients will ultimately meet these(130). Furthermore there is an on-going lack of donor organs and as such many patients who are eligible for lung transplant will die before donor organs become available. Although offering a "cure" for CF lung disease, lung transplant does not treat any of the other complications of CF and the donor organ has a limited lifespan with the ten year survival following transplant being around 50%(131). Further to these problems, transplantation requires immunosupression with drugs that as well as suppressing the immune system can lead to host damage such as renal failure and malignancy. Nevertheless lung transplantation offers a viable life changing treatment for patients with end stage CF lung disease.

1.7 Treatment of The Basic Defect in CF

Treatments for CF lung disease are available but these slow the progression of lung disease rather than halting the progression of disease altogether. In order to alter the course of disease definitively, treatments that correct the basic defect in CF are required. These broadly fall into three categories: gene correction therapy; ion channel potentiators and inhibitors (both to CFTR and ENaC); and treatments that process faulty CFTR to active protein.

1.7.1 Gene Therapy for Cystic Fibrosis Lung Disease

Following the sequencing of the CFTR gene in 1989, gene therapy was suggested as a possible curative treatment for CF due to its monogenic nature. Moreover, heterozygotes have a normal phenotype in spite of having only 50% of CFTR expression suggesting that full replacement of the gene would not be necessary for disease modification. Furthermore it has been demonstrated that only 5% transfection efficiency of CFTR is required to correct the chloride transport abnormality in CF epithelium(132), and therefore only partial correction will be required to alter the disease process in the CF lung. The first trials of gene therapy for CF took place in the early 1990s(14, 15) and now over 30 clinical trials of CF gene therapy, mainly as proof of principle, have been published(133), the efficiency of gene transfer being poor in most cases (although as noted above, inefficient gene transfer may be adequate for disease modification). A wide range of viral and non-viral vectors have been used for gene transfer, although viral administration may be impaired by a host immune response following repeat administration(134). As gene expression following viral vector administration is generally short lived this requires repeat dosing, which is likely to lead to diminished transduction efficiency(135). Successful viral gene therapy for CF may depend on the use of more sophisticated viral vector technology to attain longer gene expression. Recent strategies have included an HIV based vector in an Ebola virus derived envelope resulting in efficient and sustained gene transfer to epithelial cells(136).

In view of the present limitations to viral gene therapy for CF, non-viral vectors have also been used to effect successful gene transfer to the CF respiratory epithelium in the nasal cavity with effective gene expression (137-139), these studies were carried

out by a number of groups based in the UK and ultimately led to the formation of the UK Cystic Fibrosis Gene Therapy Consortium. Non-viral vectors have the potential to be less immunogenic and as such be more amenable to repeat dosing regimens (133). Studies to date have used cationic lipids complexed with DNA to transfect respiratory epithelium in the nasal cavity and demonstrated transgene expression following treatment. Alton et al also demonstrated an ability to transfect respiratory epithelium in the lung in the same patients with a correction in chloride transport, although this was accompanied by systemic side effects including flu like symptoms(137), although the side effects of therapy may have been related to the plasmid DNA rather than the lipid vector. In view of the potential treatment benefit of correcting the underlying physiological defect in CF with gene therapy the UK CF gene therapy consortium is embarking on the first lung multi-dose CF gene therapy trial using a non-viral vector in the near future following recent success in administration of such products to a large animal model(140).

1.7.2 Ion Channel Modulators

An alternative approach to CF treatment is to either increase chloride transport by non-CFTR channels or block the over activity of ENaC which has been demonstrated to have a potential role in disease pathogenesis. Amiloride has been utilised as an ENaC blocker but has a short half-life, which has led to newer longer acting sodium channel blockers such as PS552 being developed(141). Combined activation of calcium activated chloride channels and inhibition of epithelial sodium channels may be achieved by stimulating ATP-activated purigenic receptors (P2Y2 receptor pathway) with synthetic nucleotides(142) and although further clinical studies are

required early clinical trial evidence shows such agents to be well tolerated as well as having potential benefit on lung function(143).

1.7.3 CFTR Mutation Corrector Therapy

Several approaches have been suggested to correct the faulty gene products of several CF mutations. One such example is the use of high dose aminoglycoside to correct mutations of class 1 CFTR mutations due to premature stop codons as has been demonstrated in vivo in the nasal epithelium(144), although the practical application of this intervention may be limited by the toxic nature of this class of drug, and furthermore as the majority of patients have class II mutations the generalisibility of these treatments is questionable. Toxicity may be less prominent with a newly described compound PTC 124 (145, 146). This compound was demonstrated to improve chloride transport in the nasal epithelium in patients with class 1 mutations, although the results from this trial were not consistent when the patients received repeat dosing of the compound(147). Further research has also focused on the potential correction of class 2 mutations using the compound Sodium-4-phenylbutyrate (PBA), which acts by stopping the degradation mutant CFTR(148). This area remains a focus of on going research and may ultimately compliment other treatment approaches tailoring therapy specifically to a patient's genotype as well as phenotype.

1.8 Measuring Outcome in Trials of CF Therapy

Defining the success of any (new) therapy in CF is difficult. CF is a relatively uncommon disease, and as such the appropriate powering of clinical trials can be difficult, particularly if looking at the effects of treatment on long-term outcomes such as mortality, which may lead to potentially successful therapies being rejected inappropriately. Therefore, surrogate markers of the success of therapy are required to allow smaller numbers of patients to be utilised in studies whilst allowing them to be powered appropriately. As CF lung disease is characterised by excessive airways inflammation, one would expect any new treatment of CF lung disease, if it is to be successful, to have a beneficial effect on lung inflammation, and as such the measurement of inflammatory mediators may offer potential surrogates. This should be true for any new therapy whether it be gene correction therapy, a new antibiotic or a new agent to increase mucociliary transport. Unfortunately at present there is no universally accepted measurement of inflammation in CF and therefore the majority of studies use lung function as their primary outcome measurement as a marker of disease severity.

1.8.1 Lung Function as an Outcome Measurement in CF Therapy

The measurement of airways physiology by spirometry is well described in many respiratory diseases. FEV_1 (forced expiratory volume in 1 second) and FVC (forced vital capacity) are simple to measure and easy to perform in the clinic. FEV_1 can predict outcome in patients when used to classify the severity of lung disease in clinical studies(149). Furthermore an FEV_1 of less than 30% predicted is associated with a two year mortality of greater than 50% in CF(150). Section 1.6 concentrated

on the main therapeutic options in CF lung disease treatment. Treatments such as DNAse, aerosolised antibiotics, hypertonic saline and NSAIDs were all assessed by there ability to either improve FEV_1(104, 105, 107, 110, 115) or decrease the rate of decline of FEV_1(120) in their given populations. This is hardly surprising as FEV_1 represents the best predictor of mortality in CF(151, 152). The rate of decline in FEV_1 over time may be more reflective of the overall effects of therapy on lung disease than absolute changes in lung function in the short term but this strategy requires large numbers of patients and longer follow up to assess(153). New developments in lung function measurement such as inert gas washout may improve the sensitivity of lung function measurements as outcomes in CF therapy trials(154). However, the development of surrogate markers of inflammation may offer more insight into the underlying disease process whilst allowing the measurement of an outcome that is directly related to the treatment being given, and allow smaller numbers of patients to be used when assessing new therapies.

1.8.2 Biomarkers of Lung Disease in CF

A biomarker is a "*characteristic that is objectively measured and evaluated as an indicator of a normal biological process, pathogenic process or pharmacological response to a therapeutic intervention*"(155). Biomarkers should measure biological activity and can either be used as an endpoint test of efficacy or to discover new modes of biological activity. Functional biomarkers have been used in previous studies of CF therapy, such as nasal potential difference in CF gene therapy trials(137-139). However a growing area of research is the use of biomarkers of inflammation in CF airways disease to assess outcomes in future CF trials.

Inflammatory biomarkers have been sought directly from lung fluid in studies examining bronchoalveolar lavage fluid (BALF) and sputum as well as blood. Indeed lung secretions from CF patients contain large amounts of inflammatory mediators, neutrophils and neutrophil products. High concentrations of tumour necrosis factor (TNF)-α, interleukin (IL)1β, IL-6, and IL-8 are readily measurable(156).

1.8.3 Exhaled Breath Condensate

This technique involves the collection of exhaled air from the respiratory tract, which is then condensed to a liquid by cooling. It is proposed that this exhaled breath may contain micro droplets of fluid from the airways lining and thus allow a truly non-invasive means of measuring the airways fluid. Physical characteristics of exhaled breath such as pH(157) and levels of nitrite(158) can differentiate CF patients from controls. However, previous work performed by the UK CF Gene Therapy Consortium demonstrated insufficient levels of proteins in exhaled breath to allow detailed proteomic analysis (personal communication, Gordon MacGregor).

1.8.4 Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) involves the washing of segments of the lung during a bronchoscopy, an invasive procedure, and usually requires sedation or (in children) a general anaesthetic. Many of the early studies of CF lung inflammation utilised this technique(74, 87-89, 159-164) and indeed therapeutic response to interventions may be measured in this way(165-172). There are now accepted standard operating procedures for performing BAL in CF patients to investigate new treatments used by

the Cystic Fibrosis Foundation in the united states(173). Moreover, previous work performed by the UK CF gene therapy consortium and others has demonstrated the usefulness of BALF in the discovery of novel biomarkers in CF (174, 175). Less invasive approaches, such as sputum sampling, have been developed as a non-invasive alternative and may be more appropriate in groups of patients where repeated measurements of inflammation, such as in clinical trials, are required.

1.8.5 Sputum

Expectoration of sputum has been used for many years to monitor CF lung disease, as an appropriate sample for microbial culture. Spontaneous expectoration of sputum requires patients to have sufficient lung disease such that they have regular sputum production, therefore this means of sampling airways fluid may be less suited to children and those with less severe airways disease. Sputum induction with hypertonic saline has therefore been developed and has proved useful in a number of respiratory diseases, in particular asthma(176-180) where chronic sputum production is not a common feature. Indeed several studies have now been performed to demonstrate normal values of biomarkers in sputum for control populations(181, 182). Early studies focussed on the cellular component of induced sputum and have been particularly effective in airways diseases such as asthma. However the fluid phase of sputum has also been utilised to investigate protein biomarkers in sputum with great success(173, 183). The fluid phase of sputum has been employed in biomarker studies of the inflamed CF lung (99, 102, 184-199), and may also be used as an accurate measure of infection(200-202). Moreover, as well as identifying

pathogens by conventional means sputum can be utilised for molecular testing of pathogens and bacterial proteins(203, 204).

Several studies have now demonstrated relationships between sputum biomarkers and clinical outcome measurements such as lung function. Scott Sagel and colleagues have demonstrated significant correlations between FEV_1 and sputum neutrophil counts with IL-8, neutrophil elastase and matrix metalloproteinase levels in CF sputum from children (198, 199), a finding that has been replicated in adult populations (205). Recently a large retrospective study of 250 subject across multiple CF centres has demonstrated significant negative correlations between FEV_1 and spontaneously expectorated sputum markers including IL-8 and neutrophil elastase (193). Several studies have also demonstrated changing levels of sputum biomarkers such as IL-8 following treatment of CF exacerbation (102, 103). Therefore sputum biomarkers may possibly be used in conjunction with other outcome measures in the future to assess the effectiveness of new treatments in CF. However, further studies will be required to assess the reproducibility of these biomarkers in CF before they can be fully integrated as useful clinical tests, although early data remains promising(194). Table 1.1 demonstrates many of the presently measurable biomarkers in sputum.

Some methodological concerns with sputum as an appropriate source of biomarker relate to its processing. Sputum is most commonly liquefied with dithiothreitol (DTT) a reducing agent that breaks down disulphide bonds in mucins. This can interfere with some immunoassays designed to measure biomarkers and in order to

minimise variability from these sources, standardised guidelines have been published by the European Respiratory Society on the processing of sputum and measuring of inflammatory markers therein(206, 207).

Thus the measurement of biomarkers in sputum may lead to the development of clinically useful tests for the study of new therapies in CF and compliment present clinical measurements. It is likely that sputum contains many so far undiscovered markers of inflammation and as such provides an excellent medium for the discovery of new biomarkers. Furthermore these biomarkers may also allow further insights into the complicated pathophysiology of CF lung disease and provide information regarding the mechanisms of disease.

Biomarker Class	Examples
Inflammatory Cells	Neutrophil counts, macrophage characteristics
Cytokines	IL-1β, IL-6, IL-8, IL-10, IL-17, IL-23, TNF-α, TGF-β
Proteases	Elastase, matrix metalloproteinases, Cathepsin G
Anti-Proteases	α-1 antitrypsin, SLPI, elafin
Neutrophil Products	Myeloperoxidase, DNA
Anti-microbial Proteins	Lysosyme, lactoferrin, secretory component of IgA
Anti-microbial Peptides	Defensins, cathelicidin LL37
Markers of Tissue Injury	Elastin and collagen degradation products

Table 1.1 Possible Biomarkers in Sputum
This table demonstrates the abundance of potential biomarkers in CF sputum. These range from cytokines to natural defence proteins. In spite of a great number of target molecules being described few have made the transition into mainstream clinical assays. Table adapted from Sagel et al(173).

1.9 Discovery of New Biomarkers in CF

So far in this introduction I have reviewed some of the evidence relating to the pathogenesis, treatment and further the monitoring of CF lung disease. Although there is merit in using presently described markers of inflammation in the study of CF lung disease (and its treatment), it is likely that other as yet unknown biomarkers are present in clinical samples that will aid the study and understanding of the disease process. I will therefore concentrate on alternative methods of protein biomarker discovery in this section with particular reference to mass spectrometry.

1.9.1 Proteomics

The proteome is the entire compliment of proteins expressed by a genome, cell, tissue or organism. Proteomics is the study of the proteome including the basic chemistry of proteins; their function and role; the measurement of protein levels *in-vivo* and *in-vitro*; the discovery of new protein markers; and the interaction of proteins in complicated biological systems and as such employs a range of scientific and analytical techniques.

1.9.2 Proteomics in CF Lung Disease

The majority of studies investigating protein biomarkers in CF have focussed on the application of specific immunoassays to measure the level of known proteins. The discovery of new markers of CF has been attempted utilising a number of body fluids including serum, sputum and BALF, utilising proteomics techniques such as polyacrylamide gel electrophoresis and mass spectrometry.

Sloan et al observed protein biomarkers in CF sputum that appeared to change with disease severity during CF exacerbations using 2 dimensional polyacrylamide gel electrophoresis (2DPAGE), and observed of increased levels of myeloperoxidase, as well as increased levels of immunoglobulin breakdown products were described in this group of CF patients(208). Furthermore McMorran et al have now demonstrated increased levels of specific neutrophil proteins in BALF from children with CF(175), a finding replicated by the UK CF gene therapy consortium in a separate cohort of patients (174). Therefore proteomics offers a new approach for the discovery of biomarkers in CF lung disease and as such I have utilised this technique in this thesis for biomarker discovery.

1.10 Mass Spectrometry

1.10.1 Background

Mass spectrometry is the separation of proteins in a sample by molecular weight. The sample is first ionised (i.e. converted to gaseous phase) by laser or by electrospray (fine spraying of sample through a small diameter needle) and then enters a flight tube, where separation by molecular weight takes place. The weight of individual molecules is calculated from the speed of travel along the tube.

All mass spectrometers consist of three essential components: an ion source, a mass analyser and a detector. The sample is ionised in the ion source and these ions are then separated in the mass analyser (in most cases a flight tube, but other methods include quadropole magnets) based on their mass-to-charge (m/z) ratios and then detected by the detector (normally an electromagnetic plate that the ions collide

with). This allows the production of a mass spectrum, which represents a plot of ion abundance vs. m/z. In laser ionised mass spectrometry the ion source, mass analyser and detector are situated inside a high-vacuum chamber and as such are expensive to maintain. Electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) represent the most common ionisation methods used in mass spectrometry and produce intact protonated molecular ions of proteins and peptides i.e. the proteins being studied are generally not degraded before detection. Matrix (or energy absorbing molecules) is used to absorb the energy of the laser source and allow ionisation of the sample in MALDI TOF. Surface enhanced laser desorption/ionisation (SELDI) is a modification of MALDI TOF and will be described in the following sections.

1.10.2 MALDI TOF Mass Spectrometry

As briefly described above MALDI TOF is a form of mass spectrometry utilising a UV (laser) absorbing organic acid as matrix, and a UV laser source to desorb and ionise the proteins. The matrix is added in to the analyte molecules in excess (a saturated solution) so that the analyte and matrix co-crystalize thus increasing the surface area for interaction with the laser. This technique was first described in 1988 by Karas and Hillenkamp(209). The matrix has a number of important roles: firstly the large excess of matrix separates the analyte molecules from each other reducing intramolecular interactions; secondly the matrix absorbs large amounts of energy from the incoming photons produced by the laser causing the matrix-analyte mix on the chip surface to explode sending these molecules into the gas phase; and thirdly

the matrix donates protons to the proteins in the analyte during the gas phase (ionisation), thus giving the proteins energy to separate in the flight tube.

After the ions are formed by MALDI the protonated molecules are accelerated out of the source under the influence of a strong electric field. Once in the flight tube the ions all have the same kinetic energy thus the arrival time of a given ion at the linear detector will vary depending on its mass. The equation $E = {}^1/_2\ mv^2$ is central to mass spectrometry. E is the kinetic energy of an ion, m mass and v is velocity. Ions of the same charge are accelerated to the same energy and therefore their velocity (and therefore time of flight) will be inversely proportional to the square root of their molecular mass, and therefore heavier ions travel slower than lighter ones. Time of flight (TOF) mass spectrometers can be either linear and reflector type instruments.

Reflector instruments use an ion mirror. The ion mirror (not a physical mirror) uses high voltage to stop ions in the flight tube and then reaccelerate them towards the detector allowing better resolution. Reflector-type instruments allow better resolution of peptides and allow the monoisotopic mass of peptides and proteins to be calculated, whereas linear TOF measures the average mass of the combined isotopes of the peptide or protein. Surface enhanced laser desorption time of flight (SELDI TOF) is a modification of a linear MALDI TOF instrument with the addition of on-chip chromatography.

1.10.3 SELDI TOF Mass Spectrometry

SELDI TOF MS is a combination of affinity capture technologies with mass spectrometry and is based on the initial work of Hutchens and Yip(210), and in particular the development of Surface Enhanced Affinity Capture (SEAC). In contrast to MALDI where the chip surface merely acts to present a sample to laser desorption, surface enhanced technology utilises a chip surface that plays an active role in extraction, fractionation, clean up or amplification of the sample. Importantly the surface enhanced affinity capture element of SELDI TOF MS takes place prior to the actual mass spectrometry. Samples are processed on the chip surfaces with a number of buffers and washing steps prior to subsequent MS. This technology was commercialised as the Ciphergen ProteinChip (Ciphergen Biosystems, Freemont, California) in 1997. Protein chips are pre-coated stainless steel slides, with the coating determining the surface properties. Each Protein Chip slide has 8 individual spots for the application of a sample and are disposable following single use. SELDI TOF MS then uses a linear time of flight instrument to calculate the average molecular weight (see above) of the proteins in the sample, and as such the mass accuracy of SELDI TOF MS instruments is not as good as true MALDI TOF instruments. SELDI TOF MS provides semi-quantitative data on the protein profile of a given sample and thus comparisons between disease groups and controls can be used. The SELDI TOF MS data may either be analysed in terms of individual significantly different peaks or using the whole protein profile to demonstrate differences between groups. Figure 1.4 is a graphical representation of SELDI TOF MS technology.

SELDI TOF MS technology has been utilised by many groups to discover biomarkers by comparing samples from disease groups to control groups. The primary focus of much of this research has been to determine diagnostic biomarkers in a wide range of diseases such as rheumatoid arthritis (211), HIV infection (212), prostate cancer (213), ovarian cancer (214, 215), motor neurone disease(216), ischaemic heart disease(217), and infectious diseases (218, 219). These studies utilised a diverse range of body fluids such as serum, urine and CSF and joint fluid. These studies vary in the number of subjects used and the type of analysis performed. Initial evidence suggested that SELDI TOF MS had the potential to become an important diagnostic platform for use in the diagnosis of complicated diseases. This work however, has not been without controversy.

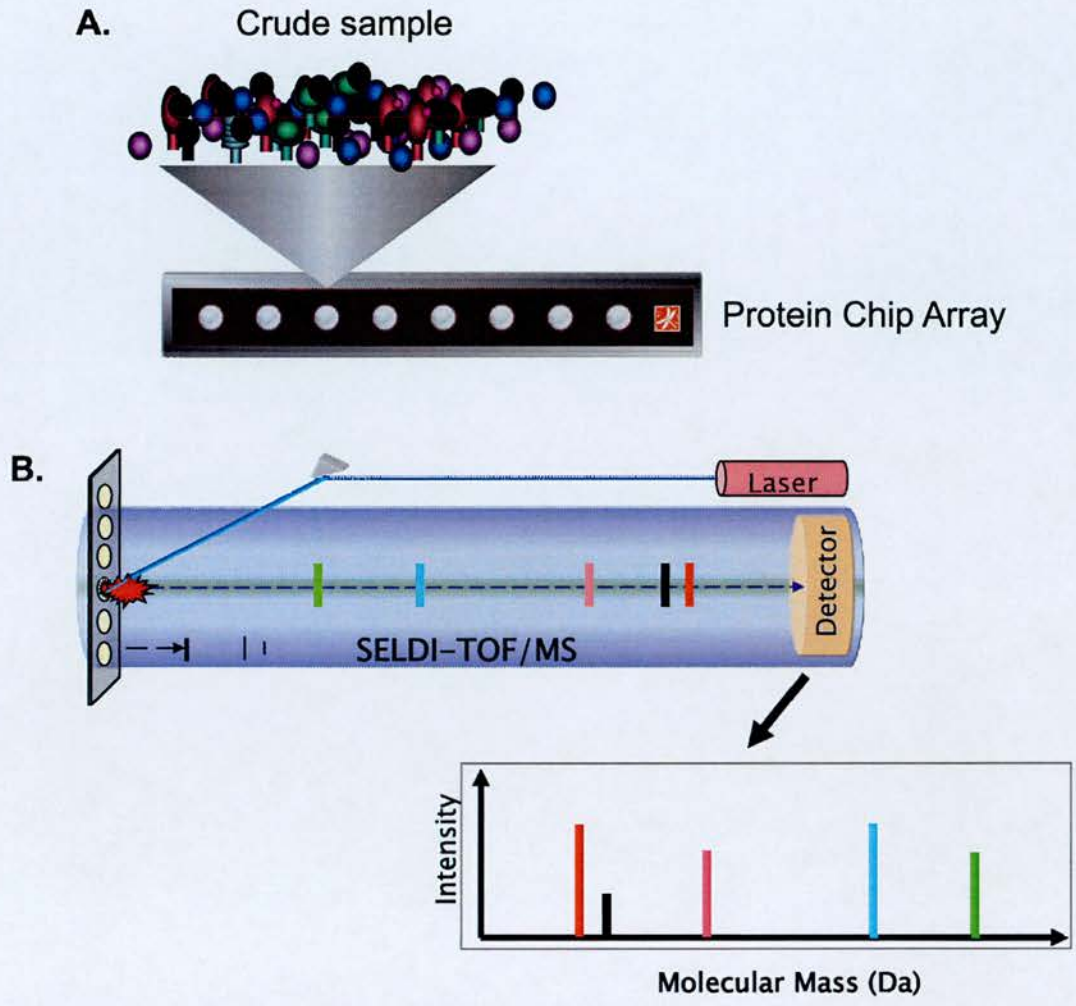


FIGURE 1.5 SELDI TOF MASS SPECTROMETRY

A. Crude sample is added to a protein chip array and processed prior to the addition of matrix. B. Sample and matrix are ionised by laser and the resultant ions fly along the vacuum filled flight tube and time of flight is calculated. Thus a representative spectrograph of the constituent proteins is generated. Adapted from Abramovitz et al(220).

1.10.4 Controversies with SELDI TOF MS

A major study utilising SELDI TOF MS published in the Lancet in 2002 led to much controversy(214). In this (Lancet) study sera from patients with ovarian cancer were compared with that of healthy controls using SELDI TOF MS. It was suggested that the resultant data could be used to differentiate cases of ovarian cancer from normal control subjects, and serve as a diagnostic screening test for ovarian cancer, which could be useful in the early diagnosis of disease (in a disease characterised by frequent presentation at an advanced stage). The data analysis however was based on the differential clustering of peptides less than 1000Da in weight, a mass range in which one would expect significant experimental noise. Further problems with the study included non-uniform sample processing prior to mass spectrometry as well as inconsistencies in the mass spectral data processing and analysing. The interpretation of the data in this particular study also relied on the examination of mass spectral patterns rather than identifying individual proteins, an approach which has now been widely criticised in the literature. Indeed further independent analysis of data sets from this study by other researchers led to completely different conclusions being drawn (221). In particular, Baggerly et al demonstrated that inconsistent data processing alters the ultimate outcome of these sorts of studies. It has now been suggested that the identification of key biomarkers following initial proteomic studies may provide a more viable route to clinical application(222), and furthermore may allow the function of such markers to be investigated. Therefore utilising SELDI TOF MS as a screening tool to identify individual biomarkers for identification may be a more valid approach than attempting to use biomarker patterns *per se* as a clinical test. SELDI TOF MS has been applied to a small

number of patients with COPD demonstrating a protein profile in BAL(223), and also in pulmonary sarcoidosis(224). The UK CF gene therapy consortium has demonstrated differential protein expression in BALF in CF, findings that have been replicated by an independent group(174, 175). There is as of yet a lack of published data utilising SELDI TOF to profile sputum in CF, although the complimentary proteomic approaches of two-dimensional electrophoresis(208), shotgun protein sequencing(225), and affinity immunoproteomics(196) have been applied to sputum in an effort to discover new biomarkers and been successful in this aim.

1.10.5 A Rational Approach with SELDI TOF MS

In view of the obvious problems with data analysis of mass spectral data I have used SELDI TOF MS as a screening platform for the selection of biomarkers prior to their confirmation with traditional proteomic techniques and then validation in further studies. In this way SELDI TOF MS is used as a non-biased, high throughput, screening tool to discover new biomarkers rather than being utilised as a primary assay that employs bioinformatics to differentiate between disease groups. Furthermore as CF is a disease with established diagnostic tests and algorithms the aim of this thesis was to discover biomarkers that may be related to the disease process and ultimately may be used to monitor the response to new therapies. Thus SELDI TOF MS analysis may be seen only as the first step in biomarker discovery and validation, which needs to be followed by biomarker identification and confirmation of biomarker expression by other techniques.

1.10.6 Protein Identification

When a clinical sample is assayed by platforms such as SELDI TOF MS, the initial analysis will reveal the molecular weight and abundance proteins and peptides in a sample but not provide the identification of significant protein peaks and thus protein identification by other methods is necessary. For example, initial analysis may reveal highly expressed proteins at 10, 12 and 14 kDa, but will give no information as to what these proteins are – this will require further proteomic analysis. Following the selection of a peak of interest this protein must then be purified by, for example, gel electrophoresis and chromatography and then subjected to trypsin digestion and identification with peptide mass fingerprinting or MSMS. Peptide mass fingerprinting is the process by which the trypsin digested fragments of a protein are subjected to mass spectrometry and the resultant masses are searched against databases of known peptide fragments and thus a theoretical sequence of a digested protein can be used to predict the parent molecule, with these databases being based on theoretical digestions of known proteins by the identified trypsin cleavage sites. Thus a database searching for protein by mass fingerprinting will give a list of potential matches and the best fit is determined by scoring systems such as the Molecular Weight Score (MOWSE), with some databases giving a statistical probability of the likelihood of a match(226). Formal identification of a protein is completed when the identity is confirmed by antibody techniques such as western blot and ELISA, or by amino acid sequencing of a protein by MSMS (for further details please refer to chapter 4). Following protein identification, immunoassays may then be employed to measure protein biomarkers quantitatively and thus applied clinically to diseases such as CF.

1.11 Other Methods of Biomarkers Discovery

As well as containing proteins, sputum in CF is likely to contain non-organic materials such as metal ions. Increased levels of total iron and iron binding proteins have been reported in the sputum of patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) in cross-sectional studies, possibly reflecting the level of lung inflammation in these patients(227-230). The measurement of metal ions in body fluids is a specialised technique but can be performed by inductively coupled plasma optical emission spectrometry. This will be covered in chapter 6 of this thesis.

1.12 Hypothesis

CF sputum contains biomarkers of disease that have yet to be discovered and utilised. I propose that by using a proteomics approach (and other approaches as appropriate) I will be able to discover biomarkers of CF lung disease and compare these to healthy control populations and patients with other lung diseases and furthermore that these may be investigated and further validated in CF exacerbation.

2.0 Materials and Methods

2.1 Reagents Used

All chemicals were purchased from Sigma (UK) unless otherwise stated.

Hypertonic Saline 3, 4 and 5% (HS)

Dulbeccos phosphate buffered saline (DPBS)

Dithiothreitol 0.1% (DTT)

Triton X100

Ammonium Acetate

Tris Hydrochloric Acid (tris HCl)

Ammonium Bicarbonate

Nickel Sulphate

Cobalt Chloride

Sinnapinnic Acid (SPA)

Alpha-cyano-4-hydroxycinnamic acid (CHCA)

Acetonitrile

Trifluoroacetic Acid (TFA)

2.2 Subject Selection for Studies

All subjects gave informed consent prior to participation in studies related to this thesis. The South East Scotland Research Ethics Service (SESRES) approved all studies.

2.2.1 Healthy Volunteers

Healthy Volunteers were recruited from university and hospital staff on the Western General Hospital campus in Edinburgh. Any volunteers with a history of respiratory disease, for example asthma or COPD, were excluded from further participation in studies.

2.2.2 Cystic Fibrosis Patients

All Cystic fibrosis patients were attending the Scottish National Adult Cystic Fibrosis Clinic at the Western General Hospital, Edinburgh unless otherwise indicated. Patients attending this clinic had a formal diagnosis of cystic fibrosis by clinical and genetic parameters. As such the population of CF patients used in the following studies represent an adult population with minimum age being 16 years.

2.2.3 Asthmatic Patients

Asthmatic patients were recruited from the adult asthma clinic at the Western General Hospital, Edinburgh unless otherwise indicated. Diagnosis had been previously confirmed on clinical grounds meeting British Thoracic Society criteria (231). This represents a highly selected group of asthmatic patients attending a secondary care clinic.

2.2.4 COPD Patients

COPD patients were recruited from the adult respiratory clinic at the Western General Hospital, Edinburgh unless otherwise indicated. Diagnosis had been previously confirmed on clinical grounds and parameters supported British Thoracic Society criteria (232).

2.2.5 Bronchiectasis Patients

Patients with bronchiectasis were recruited from the adult respiratory clinic at the Western General Hospital, Edinburgh unless otherwise indicated. All patients had a diagnosis of Bronchiectasis confirmed by Computed Tomography (CT) scan of chest.

2.2.6 Cystic Fibrosis Patients Undergoing Exacerbation

Patients with CF confirmed as above were recruited at the time of exacerbation on attendance to the Scottish National Adult Cystic Fibrosis Clinic at the Western General Hospital when they required antibiotics. The definition of exacerbation was: the clinical need for antibiotic therapy above normal treatment. The clinical need for antibiotic therapy was based on the parameters of a decrease in FEV_1, increase in cough, increase in sputum production, increase in breathlessness, or an increase in temperature.

2.3 Spirometry

Forced Expired Volume in 1 second (FEV_1) was measured according to ERS/ATS guidelines(233) using a Vitalograph spirometer (Vitalograph, Buckingham, UK) that was calibrated twice weekly. FEV_1 was recorded as % predicted.

2.4 Sputum Collection

2.4.1 Procedure

Some CF patients were able to expectorate freely without the need for induction, and if this was the case spontaneous samples were collected. In some CF patients both spontaneous and induced samples were collected. Sputum induction was carried out with hypertonic saline (HS) following a strict protocol. Prior to sputum induction subjects were pre-treated with 400 mcg of salbutamol, a bronchodilator to avoid bronchoconstriction secondary to hypertonic airways challenge. FEV_1 was measured as above and if it fell by greater than 20% during the collection then the procedure was abandoned and further bronchodilator given.

Subjects inhaled nebulised hypertonic saline solution at successive concentrations of 3, 4 and 5 % similar to a method previously described (176, 177). Saline was nebulised through a Liberty ultrasonic nebuliser (Clement Clarke, Essex, UK). Each concentration of saline was inhaled for 5 minutes. Spirometry was performed following each inhalation step and the procedure abandoned if there was a drop in FEV_1 greater than 20 %. After each inhalation period the patient was asked to blow their nose into a clean handkerchief and rinse their mouth with water. Patients were then asked to cough and expectorate sputum. This process was repeated for each

successive concentration of HS. The success rate of sputum induction varied between disease groups but was generally above 70%.

2.4.2 Sample Collection/Transportation

Sputum was collected into polypropylene tubes and transported on ice for further sample preparation. All sputum was transferred to a clean laboratory for preparation.

2.4.3 Sputum Preparation

Sputum was prepared as a modification of previously described techniques(176, 177) and processed within 2 hours of expectoration from the patient. All reagents and sample tubes were pre-chilled to 4^0C. The collected sample was transferred to a sterile Petri dish and mucus plugs were separated from saliva using forceps. Plugs were then transferred to a fresh Petri dish and the gathered together by moving the mass of plugs in circular movements around the dish until a solid mass was formed expelling any remaining saliva.

The mucus plugs as above were transferred to a pre-weighed polypropylene tube and the weight calculated on a standard laboratory balance. 4x weight in volume of 0.1% DTT (i.e. 4mls/1g of plugs) was added to the selected mucus plugs. This mixture was then mixed with a 1ml pastette and then vortexed for 15 seconds. The sample was then mixed on a rotator at 4^0C for 15 minutes. Following rotation a further 4x weight in volume of DPBS was added to the sample and it was vortexed for 15s. The diluted sample was then filtered through pre wet (with DPBS) 48µm pore nylon gauze set in a polypropylene funnel into a chilled 15ml polypropylene tube.

The above sample was then centrifuged at 1200 rpm for 10 minutes at 4^0C. The supernatant was removed for storage at -80^0C in polypropylene cryovials (Nunc, Thermo Fisher Scientific, Denmark) until further analysis.

2.4.4 Preparation of Cellular Phase

The cell pellet from above was resuspended in DPBS in a ratio of 1 ml to 0.5mm depth of pellet. Resupended cells were spun on to glass microscope slides at 400 rpm for 5 minutes. Slides were air dried, fixed with methanol and stained by May-Grunwald-Giemsa using MGG Quick Stain (Bio-Optica, Milan, Italy).

2.5 Collection and Preparation of Serum Samples

Venous blood was collected by venepuncture of the forearm. Blood was collected into serum tubes with pre-added clotting activator (monovette serum collection tubes, Sarstedt AG and Co, Germany). The tube was then mixed by inverting 5 times. Blood was left to clot at room temperature for 45 minutes. Tubes were then centrifuged at 1800g for 15 minutes at room temperature. Separated serum was removed into polypropylene cryovials (Nunc, Thermo Fisher Scientific, Denmark) and stored at -80 ^{0}C until further analysis.

2.6 Protein Assay of Sputum Samples

A protein assay of sputum samples to be used in mass spectrometry experiments (see below) was performed. Total protein content of sputum samples was determined by a Pierce reducing agent compatible BCA (bicinchoninic acid) protein assay (Thermo

Fisher Scientific, Denmark). Samples were compared against a standard curve in 0.05% DTT diluent (the final concentration of DTT in prepared sputum samples) at a range of 62.5 – 2000μg/ml. 20μl of each sample or standard were added to polypropylene tubes with 20 μl of assay reconstitution buffer. Tubes were sealed with parafilm and incubated at 37^0C for 15 minutes. Following this 1 ml of kit working reagent was added to each tube and incubated at 37^0C for a further 30 minutes. 200 μl of each sample were transferred to a microplate and read in a plate reader at wavelength of 540nm and the protein concentration calculated from the standard curve.

2.7 Surface Enhanced Laser Desorption Time of Flight Mass Spectrometry

2.7.1 Protein Chips Used

For the SELDI TOF MS experiments in this thesis four types of ProteinChip were utilised: A normal phase protein array (NP20) with no specific binding properties; a weak cation exchange (CM10) at varying pH; a strong anion exchange (Q10) at varying pH; and an immobilised metal affinity surface (IMAC) activated with nickel. CM10 and Q10 experiments were performed using a 96 well bioprocessor (Ciphergen, USA), a means of attaching wells on top of the spot surface allowing larger volumes of sample to be used, whilst processing up to 12 chips at one time. IMAC Experiments were not carried out in the bioprocessor, as metal reagents are known to bind to the bioprocessor reservoir wells and not the chip surface (personal communication Nathan Harris, Ciphergen, UK). NP20 experiments were carried out on-chip allowing smaller volumes of samples to be utilised with no pre-activation of

the surface being required. All experiments were performed at room temperature unless otherwise indicated.

2.7.2 CM10/ Q10 Chip Protocols

CM10 and Q10 ProteinChips were placed in the bioprocessor. Chromatographic spots on the chip surface were equilibrated by adding buffer: 200 µl of either 100 mM ammonium acetate/0.1% triton (pH 4/6) or 100 mM tris HCl/0.1% triton (pH 8/10) to each well. The bioprocessor was shaken at 600 rpm for 5 minutes and then emptied. This process was repeated. The bioprocessor was emptied and 20 µl of sample (sputum fluid phase) was added to a well with 230 µl of the appropriate buffer/0.1%triton. The plate was sealed with adhesive plate sealer and shaken at 600 rpm for 2 hours. Following this the bioprocessor was emptied and 2 washes of 5 minutes were performed with the appropriate buffer that was used to equilibrate the chip in 0.1% triton on the shaker. Following this 3 washes with buffer alone minus triton were performed. A final wash of 280 µl deionised water was then performed without shaking. Following this the bioprocessor was opened and chips were removed and allowed to air dry. 0.8 ul of sinapinnic acid matrix (in 50% acetonitrile/0.5% trifluoroacetic acid) was then added to each spot and allowed to air dry. This process was repeated. Chips were then read on a Protein Biology System 2 (PBS II) mass spectrometer (Ciphergen, Freemont, CA, USA).

2.7.3 IMAC Nickel Chip Protocol

10ul of 100mM nickel sulphate was added to each spot on an IMAC ProteinChip and incubated in a humidified chamber for 15 minutes. Each spot was then washed with

10 ul UPH_2O. A further 10ul of 100mM nickel sulphate was added and incubated as above or a further 15 minutes. Spots were washed again with water and excess moisture removed from the chip using fine tissue paper. 6ul of 0.5M sodium chloride/0.1%Triton was then added to each spot. 2ul of sample (sputum supernatant) was then added to each spot. Chips were then incubated for 2 hours in a humidified chamber. Following this spots were washed twice with 10ul 0.5M sodium chloride in PBS/0.1%Triton and then twice with 0.5M sodium chloride in PBS with no triton. A final wash was performed with deionised water. Excess moisture was removed from spots and chips were allowed to air dry. 0.8 µl of sinapinnic acid matrix (in 50% acetonitrile/0.5% trifluoroacetic acid) was then added to each spot and allowed to air dry. This process was repeated. Chips were then read on a PBS II mass spectrometer.

2.7.4 NP 20 Chip Protocol

NP 20 chips required no pre-activation. 2 µl of sample were added to spots and allowed to air dry. Spots were washed twice with 10 µl deionised water and allowed to air dry. 0.8 µl of sinapinnic acid matrix (in 50% acetonitrile/0.5% trifluoroacetic acid) was then added to each spot and allowed to air dry. This process was repeated. Chips were then read on a PBS II mass spectrometer.

2.7.5 SELDI TOF Mass Spectrometry

All SELDI TOF mass spectrometry was performed on a Protein Biology System 2 (PBS II) mass spectrometer (Ciphergen, Freemont, CA, USA) situated in the Molecular Medicine centre Laboratories of Edinburgh University. When performing

analysis on sputum samples a standardised protocol was used to read all chips (regardless of surface properties) to enable direct comparisons to be made within and between groups. The mass spectrometer was calibrated regularly with known protein and peptide standards.

Chips were placed in the mass spectrometer and read automatically with the following settings unless otherwise stated. Laser intensity of 205 with deflector set at 4000 Da and a focus mass of 7500 Da with optimisation of the instrument from 3000 to 25000 Da. Chips were read in the mass range 0-50000 Da unless otherwise indicated. Chips were read at 12 individual areas on each spot with 8 laser shots at each spot being recorded, allowing an average of 96 shots for each spot to produce mass spectra. Prior to reading each area was given two warming shots at higher laser intensity, to initiate ionisation, which were not included in the average spectra. Data were exported from the mass spectrometer to a dedicated PC and analysed in platform specific software (Ciphergen ProteinChip and Ciphergen Express, Ciphergen, Freemont, CA, USA).

When undertaking individual experiments such as protein identification a manual chip reading protocol was used. This allowed the alteration of laser intensity, detector sensitivity, focus mass and deflector settings. Individual settings will be described in the text when applicable. During manual reading, data were collected from at least 10 areas on a spot surface and averaged to form spectra following each experiment.

2.7.6 Mass Spectral Data Processing

Prior to comparisons of spectra the data were processed using Ciphergen ProteinChip software and Ciphergen Express software. Data were processed in the following fashion consistent with previously published data(174). Baseline correction was performed to enhance the contrast of peaks to baseline using a fitting width of 4 times the expected width. Noise was automatically measured from 4 to 50 kDa and spectra corrected for average noise. Data were then normalised for total ion current to account for spot to spot variability in the matrix crystallisation and efficiency of ionisation. The total ion current for an individual spectrum was divided by the average total ion current over all spectra and thus each spectrum was awarded a normalisation coefficient. A normalisation coefficient of 1 reflected individual AUC the same as average, <1 suggests a greater AUC than average, and >1 a smaller AUC than average. Thus spectra with high normalisation coefficients were individually scrutinised and, if they reflected poor quality (inconsistent or noisy signal) or absent spectra, were excluded from analysis consistent with previously published work(174). Finally peaks were detected using a peak detection wizard, which automatically detected peaks by signal to noise ratio. Peaks with a signal to noise ratio >3 were selected to perform analysis within and between groups.

2.7.7 Mass Spectral Data Analysis

Data analysis of SELDI TOF MS data was performed using the platform specific software Ciphergen Express. Following uniform processing steps as above data were subjected to a cluster wizard. This automatically clustered peaks across all spectra of a similar molecular weight (within 0.3%) allowing the comparison of biomarkers between spectra and ultimately between groups. Clusters of peaks were compared

between groups by signal intensity of the peaks. Statistical analysis between groups was performed using Mann Whitney testing. Following initial data analysis p values were corrected for multiple comparisons using the Bonferoni correction. Data were also subjected to principal component analysis to demonstrate differences between disease groups when complete protein profiles were compared using an automated algorithm on Ciphergen Express, as a means of distinguishing between groups by comparison of the overall mass spectral data.

2.8 One Dimension Polyacrlyamide Gel Electrophoresis (1D PAGE)

All 1D PAGE was performed in the Novex (Invitrogen, Paisley, UK) gel electrophoresis system. Precast 18 % tricene/glycine gels (Invitrogen, Paisley, UK) were used unless otherwise stated. MultiMark (Invitrogen, Paisley, UK) markers were used. Sputum samples were prepared 1in1 with running buffer to a final volume of no more than 20 µl and heated at 85^0C for 2 minutes. Samples were then added to gels and run at 125 volts for 90 to 120 minutes until the running buffer was seen to approach the lower edge of the gel. Gels were fixed in 50% methanol/10% acetic acid in UPH_2O for 10 minutes and then stained with coomassie or subjected to blotting.

2.9 Western Blotting

All Western Blotting was performed in the Novex Blotting Module (Invitrogen, Paisley, UK). Gels were blotted to PVDF in Tris-Glycine transfer buffer with 20% methanol at 25 volts for 1 hour. Positive blotting was confirmed by staining

membranes with Ponceau S in 7% TCA. Membranes were then incubated overnight in 5% Skimmed Milk/0.2% tween in PBS at 4^0C. The following day primary antibody (usually 1 in 1000) in PBS/0.2% tween was added to membranes and incubated at room temperature for 1 hour. Membranes were then washed once for 15 minutes and then 3 times for 5 minutes in PBS/0.2% tween. Secondary antibody was then added and incubated for 30 minutes at room temperature. Washes as above were then performed. ECL plus detection reagent (Amersham Biosciences, UK) was used as per manufacturer's instructions and the membranes exposed to xray film for 30 s, 1 minute, 5 minutes and 10 minutes.

2.10 Protein Identification

Protein identification was performed on sputum samples following purification on either 1D PAGE or a combination of pre-fractionation and 1D PAGE.

2.10.1 Protein Pre-Fractionation Using Reverse Chromatography Beads

Fractionation was carried out using HyperD ceramic reverse phase chromatography beads (Pall, New York, USA). Beads were first equilibrated in 50% acetonitrile overnight. The following day 30 µl of bead suspension is added to 4 epindorf tubes and washed twice with 1ml 10% acetonitrile/0.5% TFA in deionised water for 5 minutes. Samples to be fractionated were adjusted to contain 10% acetonitrile and 0.5% TFA i.e. 900 µl of sample with 100 µl acetonitrile and 5 µl TFA. Tubes containing the beads were centrifuged and the supernatant discarded. 250µl of sample was added to each tube and the beads/sample mixed on a rotating mixer for 30 mins at room temperature. Following this the samples were centrifuged at

300rpm for 2 mins and the supernatant removed. The supernatant representing the unbound fraction, was dried in a Speed Vac (Eppindorf, Hamburg, Germany). Following this, beads from all 4 tubes were pooled and 500ul of 10% acetonitrile/0.1% TFA in deionised water is added. The sample was mixed for 5 minutes and centrifuged at 300 rpm for 2 mins. The resulting supernatant was removed and dried Speed Vac. Following this 500 µl of 20% acetonitrile/0.1% TFA was added to the beads, mixed for 5 minutes then centrifuged and the supernatant removed and dried as above and so on. This process was repeated with increasing concentrations of buffer at from 30-70% acetonitrile/0.1% TFA. At each stage 20µl of supernatant was saved for SELDI TOF MS analysis allowing the protein profile in each sample to be recorded. Once samples were dried in the Speed Vac they were stored at -20°C prior to further analysis. Desiccated samples were reconstituted for SDS PAGE using 20 µl sample running buffer. If samples were acidic following reconstitution ammonium bicarbonate was added to achieve neutral pH.

2.10.2 SDS PAGE For Protein Identification

SDS PAGE was performed as in section 2.8 for both pre-fractionated samples and non-fractionated samples. Following staining bands of interest were excised with a clean scalpel blade and subjected to passive elution and in-solution digestion, or in gel digestion with trypsin. Passive elution prior to in solution digestion allows the visualisation of protein profiles on SELDI TOF MS from an excised band, allowing the appropriate proteins to be selected for trypsin digestion, whilst however yielding a lower concentration of protein for trypsin digestion.

2.10.3 Passive Elution From Coomassie Stained Gels

Individual gel bands were first de-stained by excising as above, cutting into smaller pieces and placing in tube with 200ul of 50% methanol/10% acetic acid. This was mixed on a rotating mixer for 30 minutes. Following this the supernatant was removed and a further volume of methanol/acetic acid was added. This step was repeated until the gel pieces were clear of stain. Once clear, 100 µl of acetonitrile was added and the sample mixed for 5 minutes, or until the gel pieces turned white. Following the removal of supernatant the gel pieces were dried in a Speed Vac (Eppindorf, Hamburg, Germany). 70 µl of 50% formic acid/25% acetonitril/15% isopropanol/10% H_2O was added and the sample was vortexed for 2 hours. The resulting elute was then used for SELDI TOF MS or subjected to in-solution trypsin digest.

2.10.4 In Solution Trypsin Digest

Passive elution material was dried as above and then reconstituted in saturated ammonium bicarbonate solution diluted 1 in 25 and again dried as before. Following this 20 µl of 10 ng/ml of proteomics grade trypsin in 25 mM ammonium bicarbonate/10% acetonitrile was added. The sample was vortexed and then incubated at 37^0C for 2 hrs. The resulting digest was then subjected to SELDI TOF MS.

2.10.5 In Gel Trypsin Digest

Gel bands were first excised and diced as above. The diced gel pieces were de-stained by incubating at room temperature in 100 µl of water for 10 min and washing

with 50 µl of acetonitrile 50% for 15 min twice at room temperature. Pieces were then washed in 50 µl of ammonium bicarbonate 100 mM for 15 min at RT and finally in 50µl of ammonium bicarbonate 100mM and 50µl of acetonitrile for 15 min at RT. The solution was discarded and the gel pieces completely dried as before.

The sample was then reduced and alkylated (to prevent disulphide bonds forming between cysteine residues in peptides). 40 µl of 10 mM DTT in ammonium bicarbonate 100mM was added to the dried samples and incubated at 56˚C for 45 min. This solution was discarded and 40µl of 55mM iodoacetamide/100mM ammonium bicarbonate was added and incubated in the dark for 30 min. This solution was then discarded and the pellet washed with 40 µl ammonium bicarbonate 100mM twice for 5 minutes and then 40 µl acetonitrile for 5 minutes. The solution was discarded and the gel pieces dried completely as before.

Trypsin 0.01 µg/ml in 50mM ammonium bicarbonate was added to cover the gel pieces and incubated at 4˚C for 45 min. Excess solution was then removed and 40 µl of 50mM ammonium bicarbonate was added and incubated overnight at 37˚C. After digestion, the samples were centrifuged at 300rpm for 10 minutes and the supernatant removed and kept at 4˚C. The gel pieces were then washed as follows and the supernatants added to the original supernatant. The extraction and washing steps involved: (i) 20 µl 25 mM ammonium bicarbonate for 15 min, (ii) 20 µl 25 mM ammonium bicarbonate and 20 µl acetonitrile for 15 min, (iii) 20 µl 5% formic acid for 15 min twice, (iv) 20 µl 5% Formic acid and 20 µl acetonitrile for 15 min twice. DTT was added to the pooled supernatants to give a final concentration of 1 mM DTT. The solution was dried as before in a speed vac.

2.10.6 SELDI TOF MS For Protein Identification

Peptide mass fingerprinting was performed on the (PBS II) mass spectrometer (Ciphergen, Freemont, CA, USA). All protein identification work was performed on normal phase (NP20) ProteinChips. 1 µl of digested protein (from in solution digest) was added to each spot and allowed to air dry. 0.8 µl of Alpha-cyano-4-hydroxy Cinnamic Acid (CHCA) in 50% acetonitrile/0.5% trifluoroacetic acid was added to each spot twice as matrix. Chips were read with a manual protocol as in section 2.7.4. Mass spectral data were then exported for online database searching (see below).

2.10.7 MALDI TOF MS for Protein Identification

For in-gel protein digestions, peptide mass fingerprinting was performed on MALDI TOF using a Voyager DE STR mass spectrometer (Applied Biosystems CA, USA) situated in the Department of Chemistry, University of Edinburgh. 1 µl of in gel digest material was spotted onto a stainless steel MALDI plate (similar to a ProteinChip but containing 100 spots). 1 µl of a saturated solution of CHCA in 50% acetonitrile and 0.5% trifluoroacetic acid was then added to each spot. Spots were then read manually. Spectral data were then exported to Data Explorer software (Applied Biosystems CA, USA) for processing. Processing involved internal calibration to trypsin fragments and removal of isotopic data from spectra, to leave monoisotopic peaks. Mass spectral data was then extracted for online database searching.

2.10.8 Tandem Quadropole Time of Flight Analysis

Tandem Q-TOF analysis was performed to confirm identification of some proteins using a Q-star tandem MS instrument (Applied Biosystems CA, USA) with SELDI chip interface. This allowed ProteinChips to present samples for Q-TOF and collision induced dissociation (CID). The quadropole performs separation of peptides by charge using a magnet, allowing individual peptides to be selected and separated from other peptides. The selected peptides are then released into a collision chamber where under the influence of an electric field they collide and are separated into individual amino acid constituents. All Q-TOF work was carried out by Nathan Harris, an employee of Ciphergen, at their UK training laboratory.

2.10.9 Peptide Mass Finger Printing (PMF) Using Online Data Bases

Mass spectral data of digested proteins was subjected to online searching. MASCOT (Matrix Science, Boston, USA) and MS FIT (University of California, San Francisco, USA) databases were employed for PMF data generated by SELDI TOF, MALDI TOF and Q TOF. Both databases rank possible protein identification from peptide mass fingerprint data in terms of a probability score(226) (Molecular Weight Search [MOWSE]) and confirmation can only be made either with secondary antibody mediated techniques or further mass spectrometry such as Q-TOF to provide the amino acid sequence of individual peptide fragments.

2.11 Immunoassays of Biomarkers

Immunoassay of specific protein biomarkers was carried out with Enzyme-linked immunosorbent assay (ELISA). Biomarkers for ELISA were either informed by

SELDI TOF data or selected from previously described biomarkers of CF in the literature. All assays were double antibody sandwich assays performed in a microtitre plate. When available, commercial assays were employed but only if compatible with reducing agents due to our processed sputum samples containing 0.05% DTT. Therefore all standard curves for commercial assays were performed with the addition of DTT to standard curve diluent allowing a more accurate determination of protein levels in our samples. A number of immunoassays were also employed in serum. Details of individual assays are given below.

2.11.1 Calprotectin ELISA

An in-house calprotectin ELISA was used, which has an intra-assay coefficient of variation of 5.6% (Personal communication Mags Imrie, Edinburgh). Calprotectin mono- and polyclonal antibodies and calprotectin protein standard were kind gifts of Erling Sundrehagen, Oslo, Norway. Microtitre plates (Corning, Lowell, MA, USA) were coated with 100 µl mouse anti-calprotectin monoclonal (mouse anti-human) antibody overnight at 4°C at a concentration of 40µg/ml diluted in coating buffer (KPL Gaithersburg, MA, USA). Plates were then blocked with 1% BSA for 1 hour at 37°C and the plate washed with 0.05% tween x3. 100 µl of sample was added to the plate in dilutions of 1/5000, 1/10000 and 1/50000 for sputum (0.05% DTT in PBS diluent); 1/500, 1/2500 and 1/5000 for serum (50% fetal calf serum in PBS diluent). Purified calprotectin standard was also added to the plate in the appropriate diluent for the assay being undertaken (i.e. DTT for sputum, FCS for serum) with a top standard of 100 ng/ml and limit of detection of 1.56 ng/ml. Samples were incubated at RT for 2 hrs and the plate washed x3 as before. Anti-calprotectin

(chicken anti-human) polyclonal antibody at 1 in 1000 was added and incubated for 2 hours and washed as before. 100 µl donkey anti-chicken antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, Sulfolk, UK) was added at a concentration of 1 in 250, incubated for 2 hrs and washed x3 as before. 100 µl substrate to horseradish peroxidase (KPL Gaithersburg, MA, USA) was then added and plates were incubated for 20 minutes and then read on a microplate reader at 450 nm. Concentrations of calprotectin were then calculated from the standard curve.

2.11.2 Commercial ELISA Kits

Commercial ELISA kits were employed to measure Interleukin-8 (Biosource, UK); Myeloperoxidase (Assay Designs, Michigan, USA); Vascular Endothelial Growth Factor (Quantikine, R and D Systems, Oxford, UK); Clara cell secretory protein (Biovendor, Poland). Kits were performed as per manufacturers instructions but with the addition of 0.05% DTT to the provided sample buffer thus equilibrating standard curve to the DTT levels present in native sputum samples.

2.12 Measurement of Trace Elements in Sputum

Trace element assay was performed by the staff of the Scottish National Trace Element Reference Laboratory, Glasgow, UK under the guidance of Dr Andrew Duncan. A four-point calibration was used (0, 100, 500, 1000 µg/L Cu, Fe, Zn, Mn in 1% nitric acid). Sputum samples were centrifuged at 800g for 5 minutes and 200 µL of sample was then diluted with 2 ml internal standard solution (100 µ/L yttrium in 1% nitric acid) and mixed by inversion. Internal accuracy was assessed by use of two aqueous certified reference materials TMDA 62 and TMDA 64 (Promochem

Ltd, UK). Trace element levels were measured by inductively coupled plasma optical emission spectrometry using a VISTA AX (Varian Limited, UK).

2.13 Statistical Analysis

Analysis of SELDI TOF data is covered in section 2.7, with platform specific software being utilised as stated in the text, and further analysis carried out on GraphPad Prism 4 (GraphPad, La Jolla, CA, USA). Statistical analysis of ELISA, trace metal and clinical data were performed on GraphPad Prism 4. Local statistical advice was sought from a biostatistician. Appropriate statistical testing was chosen following Kolmogorov Smirnof testing to assess normality of distribution. Normally distributed cross sectional data between two groups was assessed with Student t test, non-normally distributed data utilised Mann Whitney testing. The Bonferoni correction was applied to cross-sectional mass-spectral data to allow for multiple comparisons. For paired data, if normally distributed a paired Student t test was used, if not non-normally distributed data a Wilcoxon matched pairs test was used. Data from the trace metal assays was analysed using Kruskal Wallace followed by Dunn's test following statistical advice to allow the comparison of each trace element between all groups of diseases. For all data, $p<0.05$ was considered significant. Further statistical method is described in the appropriate chapters.

3.0 Development of SELDI TOF MS as a Screening Tool in Sputum

3.1 Background

3.1.1 SELDI TOF MS and ProteinChip Technology

A more in depth description of mass spectrometry based techniques is given in the introduction. This chapter will deal with the use of SELDI TOF MS to screen specifically for biomarkers of respiratory disease. SELDI TOF MS has previously been demonstrated as a useful research tool in a variety of body fluids, in particular serum(213-215, 217-219). The technology has also been exploited for the investigation of respiratory tract such as analysis of bronchoalveolar lavage fluid(223, 224). This chapter covers early work during which chip surfaces were selected for future experiments and the variability of the technique and its reproducibility in terms of sputum biomarker measurement assessed.

ProteinChip technology allows a wide array of surface chemistries to be employed in biomarker experiments. Different surface chemistries may compliment as well as contrast each other; however a pragmatic approach may also be required when selecting a subset of the many chemistries available, particularly in terms of generating manageable data sets and addressing financial constraints.

3.1.2 Selection of Appropriate ProteinChip Surface Chemistry

As intimated there are several available ProteinChip surfaces available. Previous work utilising ProteinChip technology in bronchoalveolar lavage fluid(174) has

demonstrated the usefulness of anionic exchange surfaces (Q10), cationic exchange (CM10) surfaces and metal affinity (IMAC) surfaces in the discovery of biomarkers from this similar body fluid. Indeed based on previous work in this research group I chose IMAC Nickel as the metal affinity surface for future experiments in sputum.

Anionic and cationic exchange could be used at a number of different pH binding conditions prior to the addition of a sample. ProteinChip technology utilises disposable chip arrays and as such can be expensive. Therefore on pragmatic grounds decided to use a limited number of chip surfaces. This pragmatism takes account of economic concerns as well as addressing the concern of multiple comparisons leading to type 1 error.

3.1.3 Comparison of Induced and Spontaneous Sputum in CF

As many patients with CF expectorate sputum spontaneously due to their underlying respiratory disease I wished to assess whether spontaneous and induced sputum from CF patients taken contemporaneously were comparable, as many CF patients are able to spontaneously expectorate and thus this form of sampling may ultimately be more relevant to CF.

3.1.4 Studies of Reproducibilty

The reproducibility of SELDI TOF MS has previously been investigated in serum with a quoted intra assay variation of 15.6 % (variability spot to spot on a ProteinChip) and an inter assay variation of 24.4% (variability between spots on different ProteinChips)(218). In urine with the intra assay coefficient of variation

ranges from 8 – 30 %(234). In saliva intra- and inter- assay coefficients of variation were 18% and 31% respectively(235). Such data take no account of biological variability and are simply an assessment of the accuracy of the experimental procedure. Therefore the reproducibility of SELDI TOF MS measurements in sputum was assessed prior to biomarker discovery experiments.

3.1.5 Aims of Chapter

The main aim of this chapter is to select the most appropriate ProteinChip surface chemistries for further experiments. This chapter will also assess the reproducibility and repeatability of SELDI TOF MS measurement in sputum. This chapter also assesses the similarites in spontaneous and induced sputum protein profiles. Furthermore the inter- and intra- assay variability of SELDI TOF MS will be investigated. Biological variation in sputum samples from CF patients will also be measured to investigate the effect of time of day on sputum protein profiles.

3.2 Methods

3.2.1 Selection of Appropriate ProteinChip Surfaces

3 separate pH gradients were performed for ion exchange surfaces and nickel sulphate activation surface for IMAC ProteinChips was utilised as this had previously been demonstrated useful in BAL fluid(174). Pools of sputum from patients with CF and healthy controls were used to assess differences between chip surfaces. Surfaces were selected quantitatelively by the largest number of peaks differentiating CF from control with a signal to noise value of 10 or more to ensure genuine peaks were selected and qualitatively based on the appearance of spectra. Pooled sample from each group (n=at least 5 subjects) was incubated on the CM10 Protein chip at pH of 4 and 6; the Q10 ProteinChip at pH 6 and 8; and the IMAC ProteinChip activated with Nickel. For full experimental methods please refer to the methods chapter. Chips were read automatically and data was processed and clustered as described in the methods section. To determine the effectiveness of each surface, the total number of clusters across all groups was determined. Spectra were also assessed qualitatively. ProteinChip binding characteristics were ultimately chosen by their ability to separate CF from control samples as well as taking into account the binding characteristics of each chip surface.

3.2.2 Comparison of Induced and Spontaneous Sputum

During sample collection from 16 CF patients we asked them to firstly provide a spontaneous followed by sputum induction as described in the methods section. Spontaneous and induced sputum sampled were then spotted onto adjacent spots on

CM10 ProteinChips activated at pH4 and then read as before and subjected to cross correlation to assess the variability between the two sampling techniques.

3.2.3 Reproducibility

All reproducibility experiments were carried out using CM10 weak cation exchange ProteinChips activated at pH 4. To assess intra-assay variability sample from a single subject with CF was spotted onto eight adjacent spots on a CM10 ProteinChip activated at pH4. The chip was read automatically and protein peaks clustered. Peaks with a signal to noise ratio of greater than 10 were selected and the coefficient of variation for each cluster was calculated on GraphPad Prism (GraphPad, CA, USA). An average intra-assay coefficient of variation was then calculated. To assess inter-assay variability sample from a single CF subject was spotted onto six individual spots on six separate ProteinChips. Chips were read and clustered as above and coefficients of variation calculated for individual clusters of peaks. To further assess intra-assay variability samples from 4 CF patient and 4 healthy controls were run on adjacent spots on ProteinChips. The resultant data was clustered and cross correlation between each individual performed. In order to assess the effects of crude biological variability on SELDI TOF MS, 4 CF patients donated paired spontaneous sputum samples from am and pm on the same day. Samples were spotted onto adjacent spots on a ProteinChip and read and clustered as before.

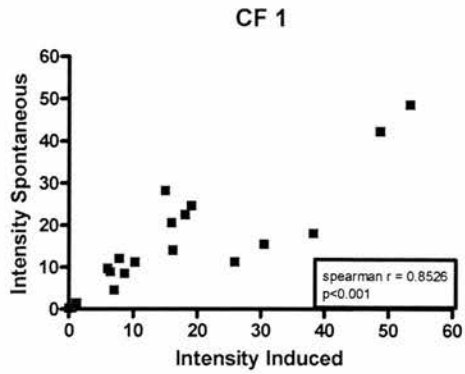
3.3 Results

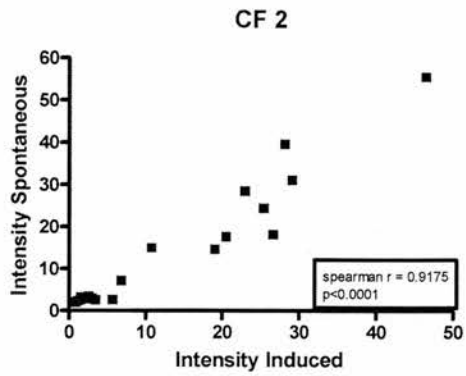
3.3.1 Chip Selection

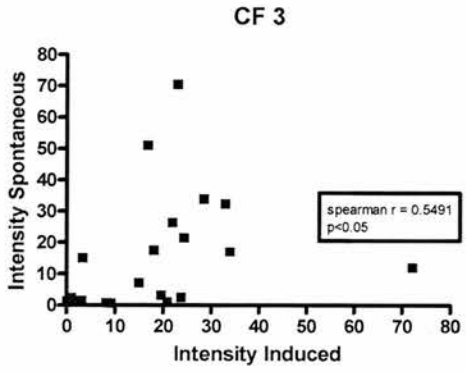
IMAC Nickel was chosen as an appropriate chip surface based on previous data form BALF fluid studies in CF(174). In pooled experiments pH4 CM10 demonstrated 107 clustered peaks vs. control compared to 92 with pH6 and thus I selected CM10 pH4 for further experiments. pH 6 Q10 revealed 80 clustered peaks compared to control vs. 75 with pH 8. In view of the similar number of peaks we chose pH8 for further experiments in order to look at proteins in the more basic range compared to the acidic range used in the CM10 analysis. These selection are broadly in keeping with the most successful surfaces for differentiating CF from control in BALF fluid(174).

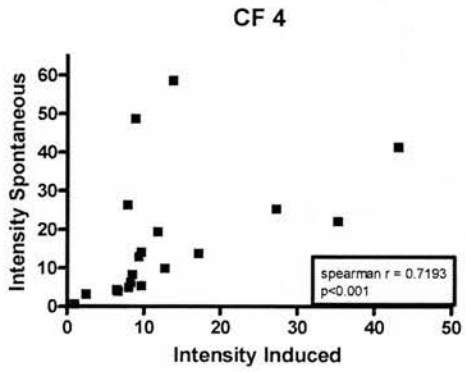
3.3.2 Induced Vs. Spontaneous Sputum in CF

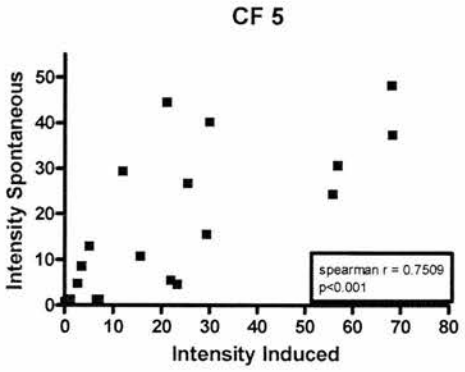
Induced and spontaneous samples were taken contemporaneously in 16 CF patients. Cross correlation of signal intensities for 19 peaks with a signal to noise ratio of >10 (to ensure we were comparing abundant peaks) revealed Spearman r values ranging from 0.55 to 0.97 all at $p<0.05$. The majority of subjects demonstrated a Spearman r >0.8 suggesting that induced and spontaneous sputum samples in CF are similar. Data are demonstrated in figure 3.1.

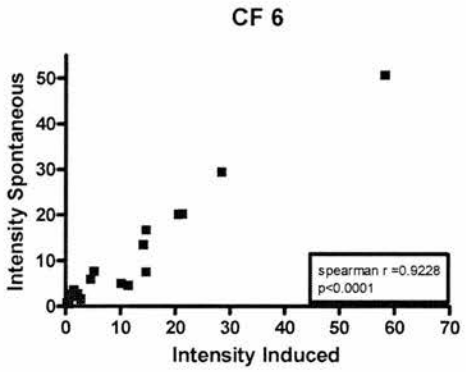

CF 1
Intensity Spontaneous
Intensity Induced
spearman r = 0.8526
p<0.001

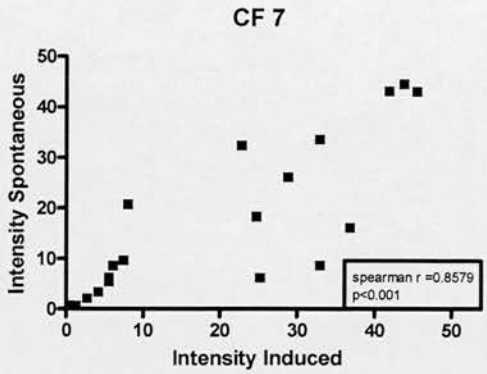

CF 2
Intensity Spontaneous
Intensity Induced
spearman r = 0.9175
p<0.0001

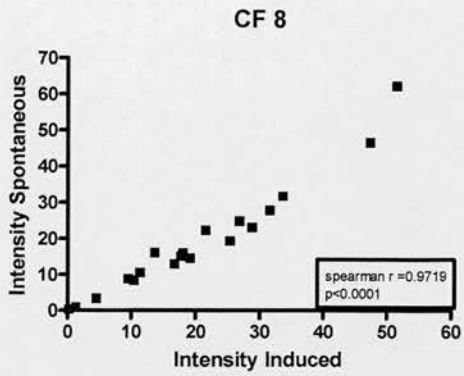

CF 3
Intensity Spontaneous
Intensity Induced
spearman r = 0.5491
p<0.05

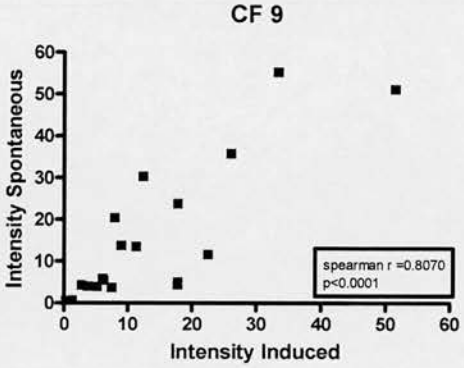

CF 4
Intensity Spontaneous
Intensity Induced
spearman r = 0.7193
p<0.001

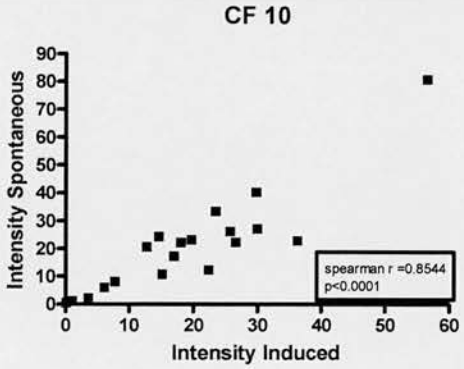

CF 5
Intensity Spontaneous
Intensity Induced
spearman r = 0.7509
p<0.001

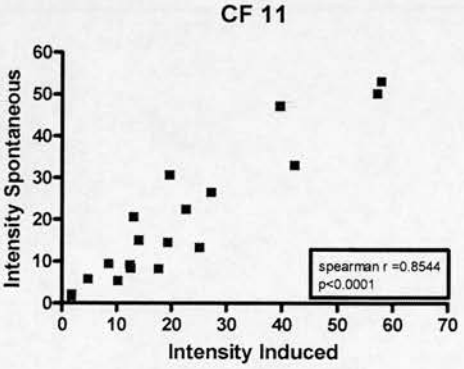

CF 6
Intensity Spontaneous
Intensity Induced
spearman r =0.9228
p<0.0001

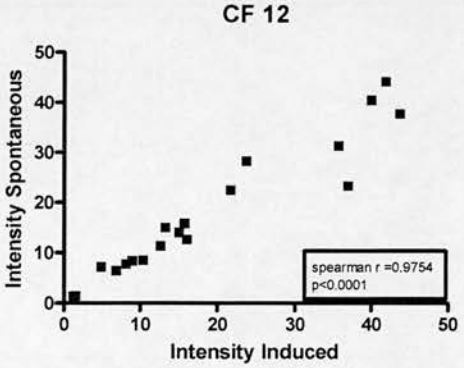

CF 7
Intensity Spontaneous
Intensity Induced
spearman r =0.8579
p<0.001


CF 8
Intensity Spontaneous
Intensity Induced
spearman r =0.9719
p<0.0001


CF 9
Intensity Spontaneous
Intensity Induced
spearman r =0.8070
p<0.0001


CF 10
Intensity Spontaneous
Intensity Induced
spearman r =0.8544
p<0.0001


CF 11
Intensity Spontaneous
Intensity Induced
spearman r =0.8544
p<0.0001


CF 12
Intensity Spontaneous
Intensity Induced
spearman r =0.9754
p<0.0001

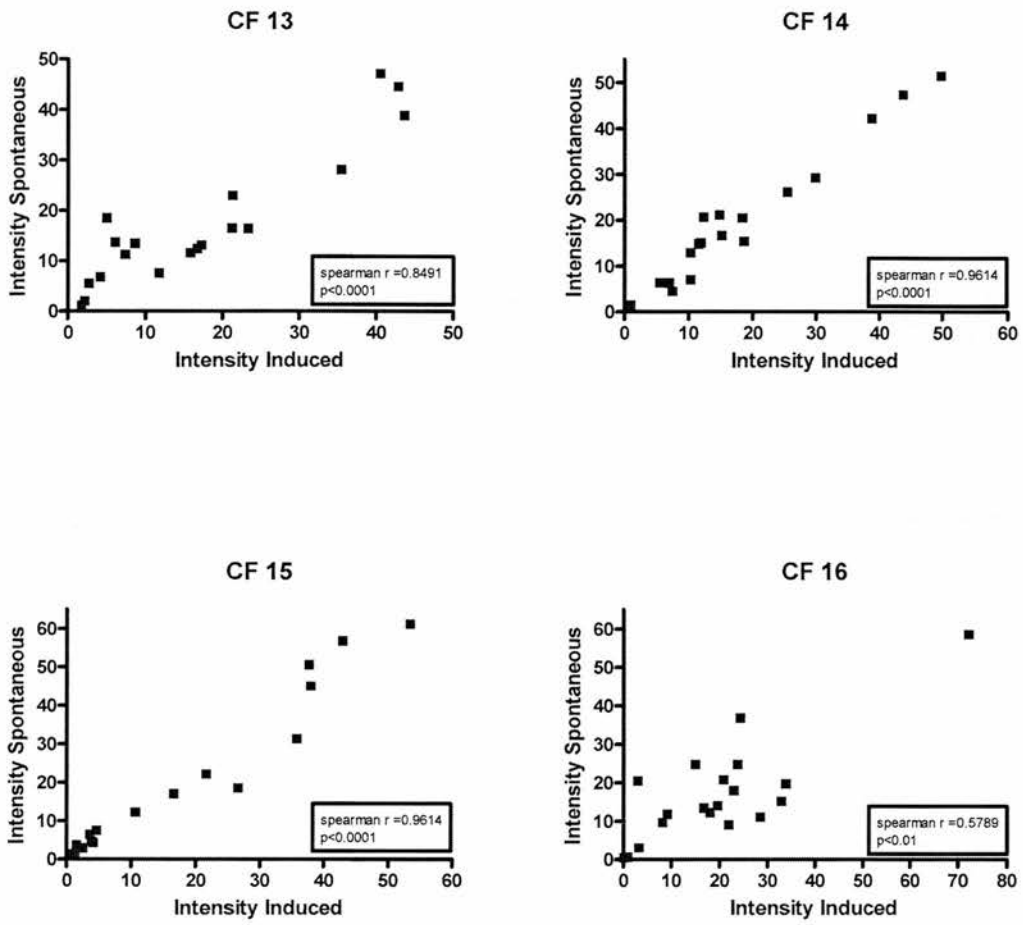


FIGURE 3.1 SPONTANEOUS AND INDUCED SPUTUM SAMPLES REPRESENT SIMILAR PROTEIN PROFILES IN CYSTIC FIBROSIS.

Spontaneous and induced samples of sputum were obtained from patients with CF. Spontaneous sputum was expectorated immediately prior to sputum induction. Samples were subjected to SELDI TOF MS using a CM10 pH 4 cation exchange surface. Peaks with a signal to noise ratio of greater than 10 were selected and the intensity of signal for each peak in the spontaneous sample was plotted against the intensity from the induced sample. Spearman r ranged from 0.55 to 0.97 and all correlations were statistically significant.

3.3.3 Intra- and Inter-Assay Coefficient of Variation

Intra-assay variation was measured by spotting the same CF sputum sample onto 8 adjacent spots on a CM 10 ProteinChip. Overlaid mass spectra demonstrating intra-assay variability are demonstrated in figure 3.2. The intra-assay coefficient of variation ranged from 11.5 to 44% with an average CV of 22.4%, which is similar to previously published data in other body fluids. Inter-assay variation over six measurements on different ProteinChips for the same sample demonstrated coefficient of variation ranging from 6 to 43% with an average CV of 16.2%.

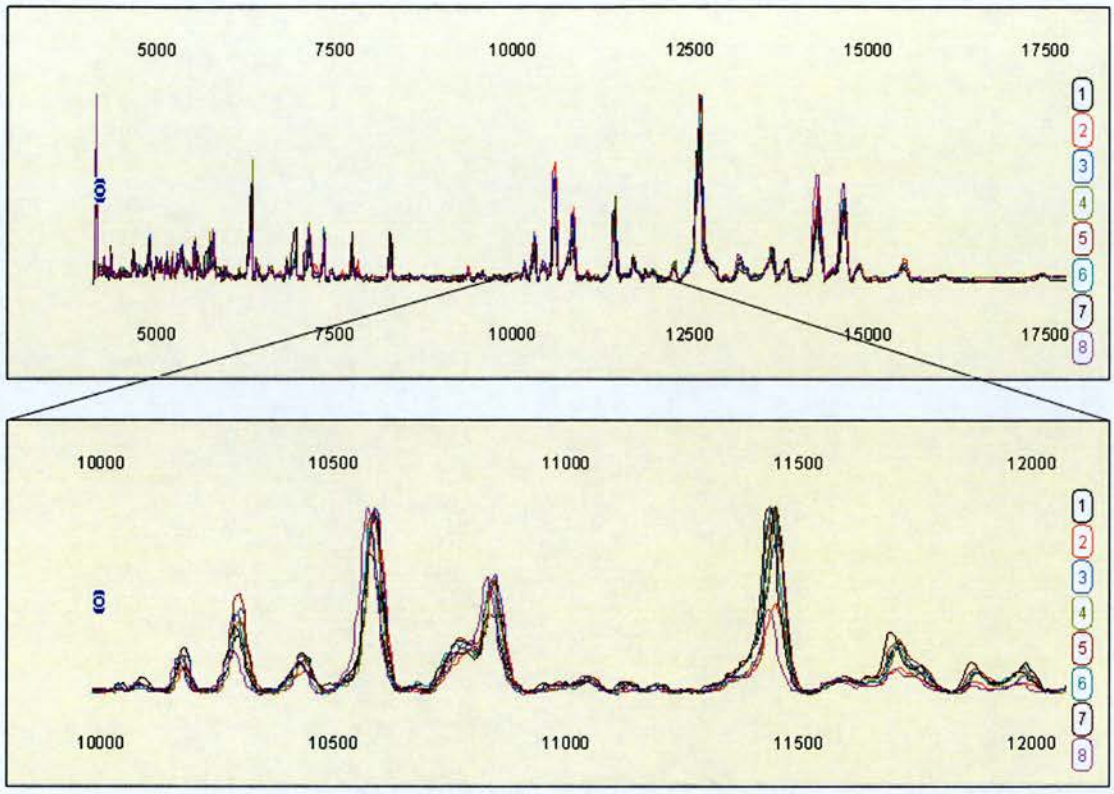


FIGURE 3.2 INTRA-ASSAY REPRODUCIBILITY OF SELDI TOF MS IN CF SPUTUM

A sample from the same subject with CF was applied to eight adjacent spots on a CM10 ProteinChip (labelled 1-8). Peaks with a signal to noise ratio of greater than 10 in the mass range of 5 to 50 kDa were selected and compared. The top panel displays the overlaid spectra for each spot from 5000-17500 Da. The lower panel demonstrates spectra in the 10000-12000 Da range. Spectra were qualitatively similar and average coefficient of variation across the spectra was 22.4%.

3.3.4 Cross Correlation of Individual Subjects to Assess Inter-Assay Variation

4 CF and 4 control subjects had sputum assessed on two separate chip surfaces in duplicate. For all correlations spearman r was greater than 0.9 suggesting highly reproducible spectra on SELDI TOF in the 5 to 50 kDa mass range. Cross correlations of individual CF subjects and controls are shown in figures 3.3 and 3.4.

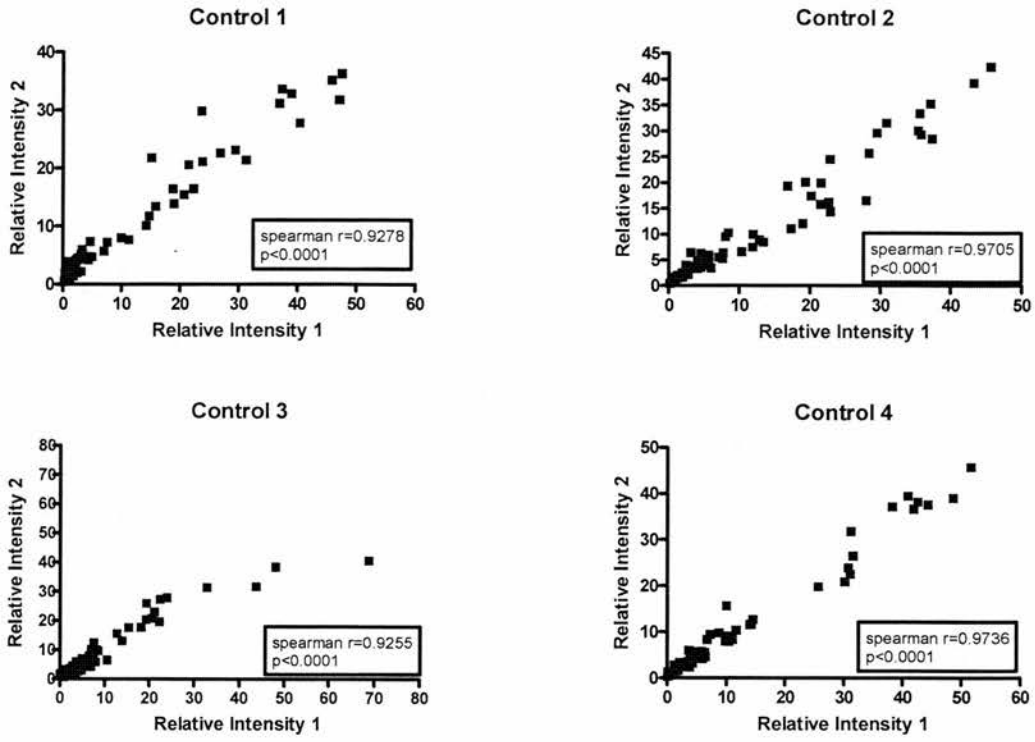


FIGURE 3.3 CROSS CORRELATION OF SELDI TOF MS IN CONTROL SPUTUM SAMPLES.
Sputum samples from 4 separate control samples were run in duplicate on two separate ProteinChips. Peaks with a signal to noise ratio of greater than 10 were selected and the intensity of signal for each peak on one chip was plotted against the intensity from the other chip. Correlation was calculated with Spearman rank testing. In all cases spearman r was >0.92 with statistical significance.

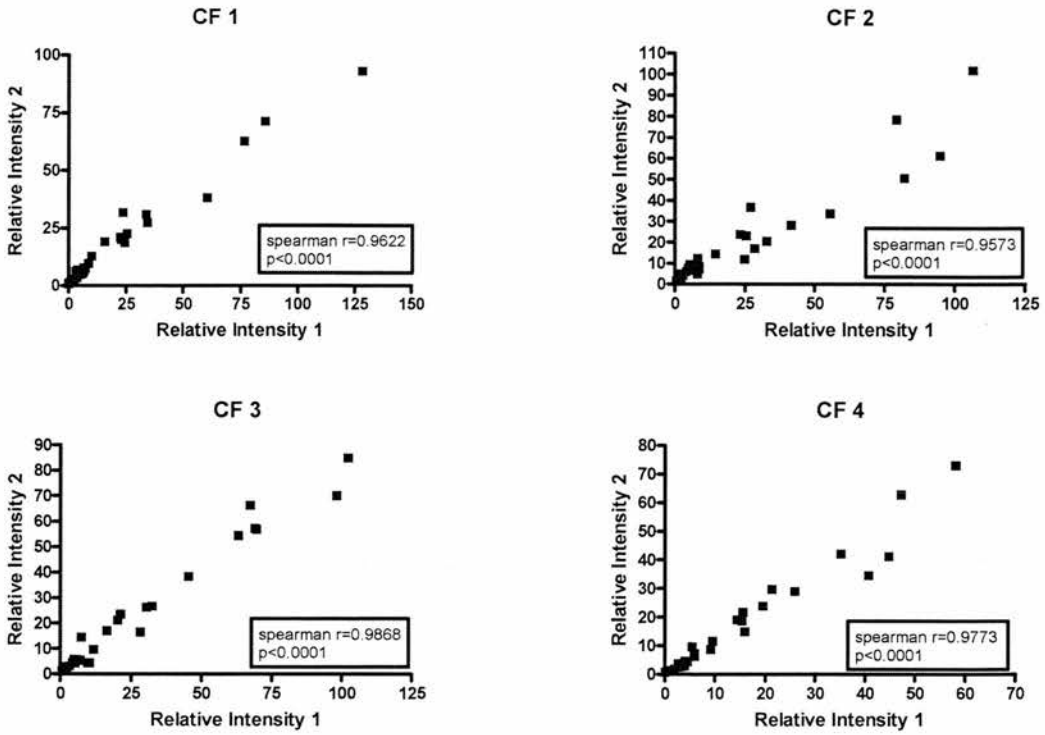


FIGURE 3.4. CROSS CORRELATION OF SELDI TOF MS IN CF SPUTUM SAMPLES.
Sputum samples from 4 separate CF patients were run in duplicate on two separate ProteinChips. Peaks with a signal to noise ratio of greater than 10 were selected and the intensity of signal for each peak on one chip was plotted against the intensity from the other chip. In all cases spearman r was >0.95 with high statistical significance.

3.3.5 Within Patient Diurnal Variability of Spontaneous Sputum

Independent sampling of spontaneous sputum from individual patients in the morning and afternoon of the same day demonstrate that the spectra are qualitatively similar and cross correlation analysis demonstrates statistically significant similarities. Figure 3.5 demonstrates cross correlations of spectral data for 4 individual patients.

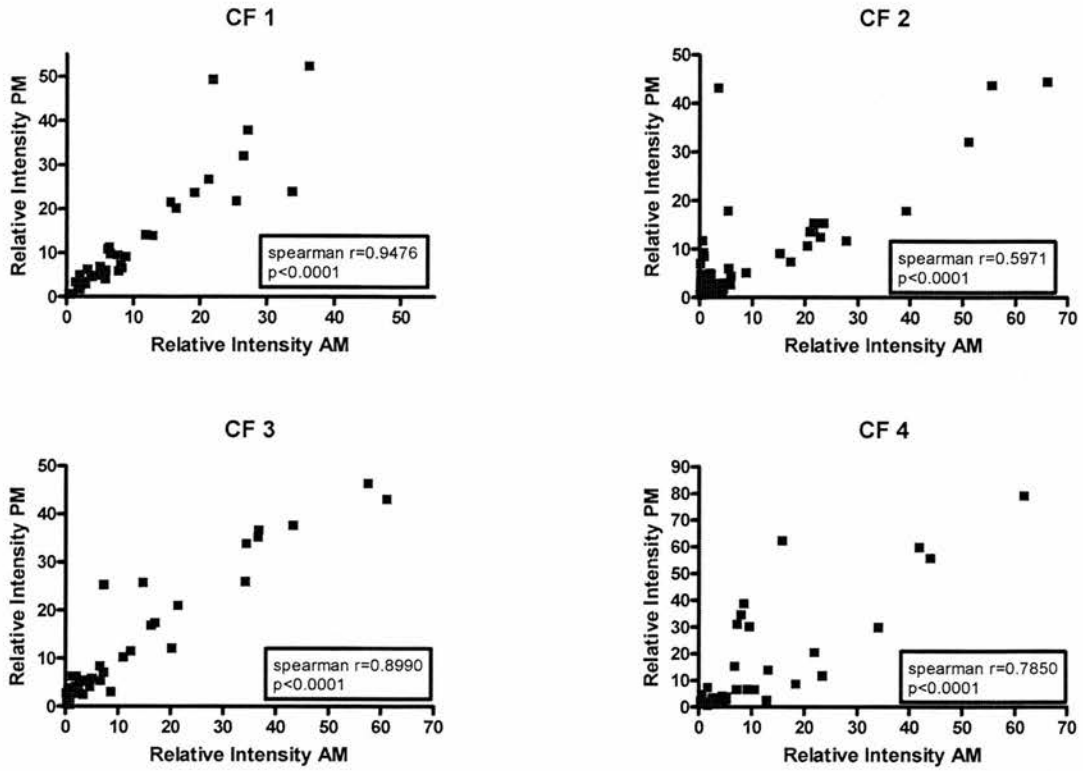


FIGURE 3.5 CROSS CORRELATION OF MORNING AND AFTERNOON CF SPUTUM SAMPLES.
Sputum samples were collected from 4 subjects with CF in the morning and afternoon of the same day and subjected to analysis on adjacent spots of a CM10 ProteinChip. Peaks of signal to noise greater than 10 in the mass range of 5-50 kDa were selected. Correlation was assessed by Spearman rank testing. Significant correlations were demonstrated for all subjects. Spearman r ranged from 0.597 to 0.948 all reaching high statistical significance.

3.4 Discussion

3.4.1 Background

This chapter deals with experiments undertaken to assess the performance and reproducibility of the SELDI TOF MS platform in the assessment of sputum samples. No previous literature existed on the application of SELDI TOF MS to sputum samples in CF prior to work performed in this thesis. I have demonstrated the criteria and rationale for the chip selections which will be utilised in further chapters as well as addressing issues of reproducibility and sample selection i.e can spontaneously expectorated as well as induced sputum be utilised.

3.4.2 Chip Selection

I selected a limited number of chip surfaces for our experiments utilising SELDI TOF MS to investigate the presence of sputum biomarkers. The reasons for this are threefold. Firstly using all of the available surface chemistry and activation combinations of ProteinChips would be very expensive. Secondly prior knowledge of the surface chemistries used in the ProteinChip arrays allows us to predict optimum performance with specific activation characteristics, for example using a very basic pH with a cationic exchange would lead to fewer proteins being bound as the majority of proteins are anionic at a basic pH. Thirdly using a greater number of chip surfaces with similar binding characteristics would lead to duplication of peak information over a number of surfaces and potentially lead to over-interpretation of any changes between CF and control groups.

Thus the pragmatic selection of three chip surfaces allows us to use a limited number of ProteinChip arrays whilst maximising the coverage of potential biomarkers. When using a cationic exchange the selection of a low pH favours the binding of more proteins as at an acidic pH most proteins would be expected to have a net positive charge and thus be attracted to the cationic exchange. The reverse is true for the anioinc exchange and thus a higher pH of activation should bind more proteins. Thus CM10 ProteinChips at pH 4 demonstrating the largest number of clustering proteins is reassuring. Q10 pH 8 although demonstrating slightly fewer proteins than pH6 was selected in view of the chemical reasons listed above, although obviously the Q10 surface at pH6 could have been chosen for the further experiments. In terms of the most appropriate metal affinity surface we selected IMAC Nickel based its previous application in CF BALF fluid(174).

Clearly the use of all available chip surfaces and activation conditions would allow a greater coverage of the complete proteome in the proteome. This may simply increase type 1 error, i.e. by measuring biomarkers over many surfaces we may find a difference between groups purely by chance. Conversely if a large number of surfaces were used any real difference may be excluded due to correcting for multiple comparisons representing type 2 error. The majority of biomarkers were found in the 5-20 kDa range, which limits the SELDI platform to the resolution of small proteins and peptides. This has led some investigators to describe SELDI TOF MS as a peptidomics rather than truly a proteomics approach, and thus one should view further data in this thesis as complimentary to other proteomic techniques rather than as a replacement for them.

3.4.3 Spontaneous and Induced Sputum in CF Patients

Following the selection of appropriate chip surfaces for the further experiments I wished to assess whether it was necessary to use induced sputum from CF subjects if spontaneous sputum was available. Many CF patients expectorate sputum spontaneously. I have demonstrated that induced and spontaneous sputum from CF patients have very similar protein profiles on SELDI TOF MS. Thus I would suggest that spontaneous sputum collection is a valid a method for sampling sputum from CF if the patient produces spontaneous sputum. These findings are consistent with previously published work demonstrating similarities in biomarker levels when measured in sputum by ELISA in Asthma(180). In patients with less severe lung disease sputum induction may be necessary but in older patients who readily expectorate sputum, induction should not be required.

3.4.4 Variability

I assessed the variability of ProteinChip technology when used to (repeatedly) determine sputum protein profiles. Intra- and inter-assay coefficients of variation were similar to those published for body fluids including serum(218). I also assessed intra-assay variability in a number of individual CF patients and controls and demonstrated significant cross-correlation. This suggests that SELDI TOF MS is a reproducible assay for biomarker discovery in sputum. However, biological variability in the CF population may lead to difficulties in the interpretation of possible difference in the CF patients from controls. Of particular concern was whether the time of day at which a sputum sample was taken could affect the protein profile present in the sputum sample, the rationale being that samples expectorated in the morning may be physiologically "older" as they result from the accumulation of

mucous and debris overnight which is expectorated in the morning, and therefore that samples collected later in the day would reflect "fresher" sputum. There are statistically significant correlations of clustered proteins when comparing samples from the same patients collected in the morning and the afternoon of the same day. This suggests that regardless of whether a sample is taken in the morning or afternoon it is likely to yield similar information in terms of protein expression. This finding is reassuring, in the context that spontaneous and induced sputum from CF patients demonstrate very similar protein profiles. Sputum sampling from CF patients can therefore be optimised to have little effect on patient lifestyle e.g. a spontaneous samples being collected in either morning or afternoon, and assessment of biomarkers by this means is likely to be readily translatable in to clinical practice.

3.5 Conclusions

In this chapter I have demonstrated selection of 3 ProteinChip surfaces with distinct binding characteristics for use in further experiments. I have also demonstrated that SELDI TOF MS may be employed to assay protein profiles in sputum with similar reproducibility to that published for serum. Furthermore I have demonstrated that spontaneous and induced sputum in CF patients have very similar protein expression profiles when measured by SELDI TOF MS and thus in spontaneous sputum producers sputum induction is not required. I have also demonstrated, albeit in a modest number of patients, that the time of day at which sputum is sampled has little effect on sputum profile.

4.0 Examination of the Sputum fluid Phase with SELDI TOF Mass Spectrometry

4.1 Introduction

4.1.1 Non-Invasive Markers of Airways Inflammation

The assessment of new therapies in respiratory disease requires realistic endpoints, particularly when considering less common diseases such as cystic fibrosis, where smaller numbers of patients will be available for the study of any new therapy. Assessment of lung inflammation using biomarkers offers the possibility to study the effects of drug therapy directly on the target organ. Traditionally direct assessment of the airway has involved invasive procedures such as bronchoscopy with endobronchial biopsy and bronchoalveolar lavage (BAL), which precludes sampling from a wide range of patients at repeated time points on ethical and safety grounds. Thus investigators have tested other means of assessing airways inflammation such as analysis of exhaled nitric oxide(236-242) and exhaled breath condensate measurements(157, 158, 243-249). Indeed in CF, exhaled breath condensate pH(157) and nitrite(158) have been demonstrated a promising surrogate markers of inflammation which change with disease severity. Measurement of exhaled nitric oxide in CF is less useful however(158). A further way in which to investigate airways inflammation is the collection of sputum, either by induction or spontaneous expectoration.

4.1.2 Assesment of Airways Inflammation Using Sputum

Sputum is the end product of lung secretions as they work their way from the peripheral to central airway, and as such is a mixture of cellular material, proteins and inorganic substances. Approximately 200 mls of sputum can be expectorated by the average CF patient in a 24 hour period(229). Several studies have demonstrated that the cellular content of sputum is mainly derived from the central airways(250, 251). In many patients sputum may be expectorated spontaneously, however the advent of sputum induction with hypertonic saline allows the collection of adequate sputum samples from a range of patient groups and control populations(179). Induced sputum cell counts and biochemical mediator concentrations have been validated in healthy adult populations(181, 182, 252). The majority of studies on induced sputum have concentrated on the cellular properties of sputum with particular reference to airways diseases such as COPD and Asthma(253), and sputum has long been used in the investigation of lung cancer. The clinical impact of sputum cytology in Asthma was best demonstrated by Green et al (176) who showed that tailoring therapy to sputum eosinophil counts reduced the number of exacerbations from which patients suffered.

4.1.3 Protein Biomarkers in Sputum

The fluid phase of sputum contains a mixture of substances related to inflammation such as pro-inflammatory cytokines. Protein biomarkers have been described in the fluid phase of CF sputum (102, 183, 194, 195, 197-199, 208, 254-259), among other diseases. These approaches mainly rely on immunoassays to detect differences in

(known) protein profiles and are as such prejudiced to proteins in a sample already known to be biomarkers.

An alternative approach is to attempt biomarker discovery i.e. looking for proteins in a sample without prejudice of which proteins should be present in a sample. This approach may utilise a number of techniques such as gel electrophoresis, mass spectrometry or multiplex protein arrays. For example sputum biomarkers of Cystic Fibrosis were investigated by 2D PAGE revealing myeloperoxidase, cleaved α_1-antitrypsin degraded IgG and interleukin-8 as biomarkers (208). SELDI TOF MS offers a further approach in proteomic biomarker discovery and has been utilised to measure protein biomarkers in CF BALF (174, 175). A full description of SELDI TOF MS can be found in the introduction of this thesis.

4.1.4 Aims of Chapter

The aim of this chapter was to utilise SELDI TOF MS to discover sputum biomarkers of lung disease in CF compared to control and other respiratory disease populations. I hypothesised that SELDI-TOF MS analysis of sputum from patients with CF, bronchiectasis, asthma, COPD and healthy adult controls would allow the discovery of novel biomarkers of CF lung disease unltimately allowing the development of clinically relevant assays to assess future treatments for CF lung disease.

4.2 Methods

4.2.1 Subjects

The South East Scotland Research Ethics Service granted ethical approval and all participants gave written consent. Sputum was obtained from 28 patients with CF, 19 with bronchiectasis, 24 with asthma, 24 with COPD, and 20 healthy controls. Patients were recruited as per criteria outlined in chapter 3.

4.2.2 Sputum Induction and Processing

Sputum induction was performed as described in chapter 2 and all samples were processed within 2 hrs of collection.

4.2.3 SELDI-TOF Mass Spectrometry

Three chromatographic chip surfaces were used to cover a wide range of protein characteristics: a weak cation exchange at pH4 (CM 10), an anion exchange at pH8 (Q10) and an immobilised metal affinity surface activated with nickel (IMAC Nickel [IMACNi]). Sputum samples were adjusted to contain 1mg/ml protein following a standard colorometric protein assay. 20 μL of sample were added to CM10 and Q10 surfaces in a bioprocessor unit (Ciphergen, Freemont, CA, USA) and 2 μL of sample was added to preactivated IMACNi surface for on spot incubation (more reproducible for IMACNi surfaces). All chips were treated with SPA matrix (2x0.8 μL/spot) and allowed to air dry. Samples were analysed on the Protein Biology System 2 SELDI-TOF mass spectrometer (Ciphergen, Freemont, CA, USA). Chips were read with a laser intensity of 205 with deflector set at 4000 Da and a focus mass of 7500 Da from 0 to 50,000 Da. A full description of SELDI TOF MS methods is given in chapter 2.

4.2.4 Data Analysis

Data were processed as outlined in the materials and methods chapter. Protein peaks were automatically clustered to identify biomarkers of similar molecular weight. Mann Whitney rank testing was performed to demonstrate statistical differences in clusters between disease groups. Values of $P<0.05$ were taken to be significant following correction for multiple comparisons with Bonferoni. Bonferoni correction was applied to the data following initial analysis and based on the number of peaks compared in a given analysis thus decreasing the possibility of a significant difference arising by chance. Therefore, following each experiment the crude p value was multiplied by the number of comparisons made and the corrected p value recorded. Principal component analysis (PCA) was performed using Ciphergen Express Software by an automated clustering algorithm using a correlation matrix allowed the entire data set to be simplified and graphed. PCA was used to display differences between groups but not for further analysis. Correlations with clinical data were performed using Spearman rank on GraphPad Prism 4 (GraphPad, Ca, USA).

4.2.5 Protein Identification

Highly abundant significantly different (between CF and control) protein peaks were selected for protein identification experiments. Pooled sputum from at least 5 subjects from either CF or control was used for identification. Protein identification work was performed as outlined in the materials and methods chapter. Commercial antibodies to calgranulin A (AbCam, Oxford, UK) and clara cell secretory protein (Biovendor, Poland) were used for protein confirmation by Western blotting.

4.3 Results

4.3.1 Recruited Subjects

Sputum was obtained from 28 patients with CF, 19 with bronchiectasis, 24 with asthma, 24 with COPD, and 20 healthy controls. Patients were recruited as per criteria outlined in chapter 3. The demographics and sputum neutrophil counts of these subjects are displayed in Table 4.1.

Group	Age Years	Sex	FEV_1 % pred	*Sputum Neutrophil %*
Asthma	47.8(2.9)	15F/8M	82.3(4.4)	*50.6(5.0)*
COPD	65.2(1.2)	10F/14M	57.7(4.1)	*79.1(2.7)*
Bronchiectasis	61.8(3.5)	15F/3M	71.7(11.6)	*83.2(5.6)*
CF	28.8(1.7)	8F/19M	59.2(3.9)	*91.6(2.0)*
Control	36.4(2.1)	11F/9M	101.4(3.1)	54.2(5.3)

Table 4.1 Subject Characteristics for SELDI TOF MS Sputum Study
Demographic, lung function, and sputum neutrophil data of the patients studied. Mean (SEM) are given.

4.3.2 Raw Mass Spectral Data

Individual spectra were produced for each subject and examined qualitatively. In terms of abundance and peak intensity the CF and bronchiectasis groups had the best quality spectra. An example of spectral data from 5 individual subjects is demonstrated in figure 4.1. Figure 4.2 demonstrates the abundance of peaks demonstrated in a subject with CF by showing the spectra at different levels of magnification. The majority of the differential peaks were found in the mass range of 4 to 20 kDa.

4.3.3 Differentiation of CF and Other Disease Groups from Control Population

Clustering and univariate analyses determined a large number of proteins present in relative proportions that differentiated between disease groups and healthy controls. Following correction for multiple comparisons with bonferoni, the number of differential proteins for each disease groups vs. control reaching pre-defined statistical significance ($p<0.05$ corrected) was as follows:

Cationic exchange (CM10): 28 CF from control, 29 bronchiectasis from control, 2 asthma from control, 2 COPD from control.

Anionic exchange (Q10): 28 CF from control, 34 Bronchiectasis from control, 13 asthma from control and 14 COPD from control.

Metal affinity surface (IMAC Ni): 46 CF from control, 48 bronchiectasis from control, 1 asthma from control and 5 COPD from control.

The total number of clustering peaks for each chip surface and disease group are demonstrated in table 4.2.

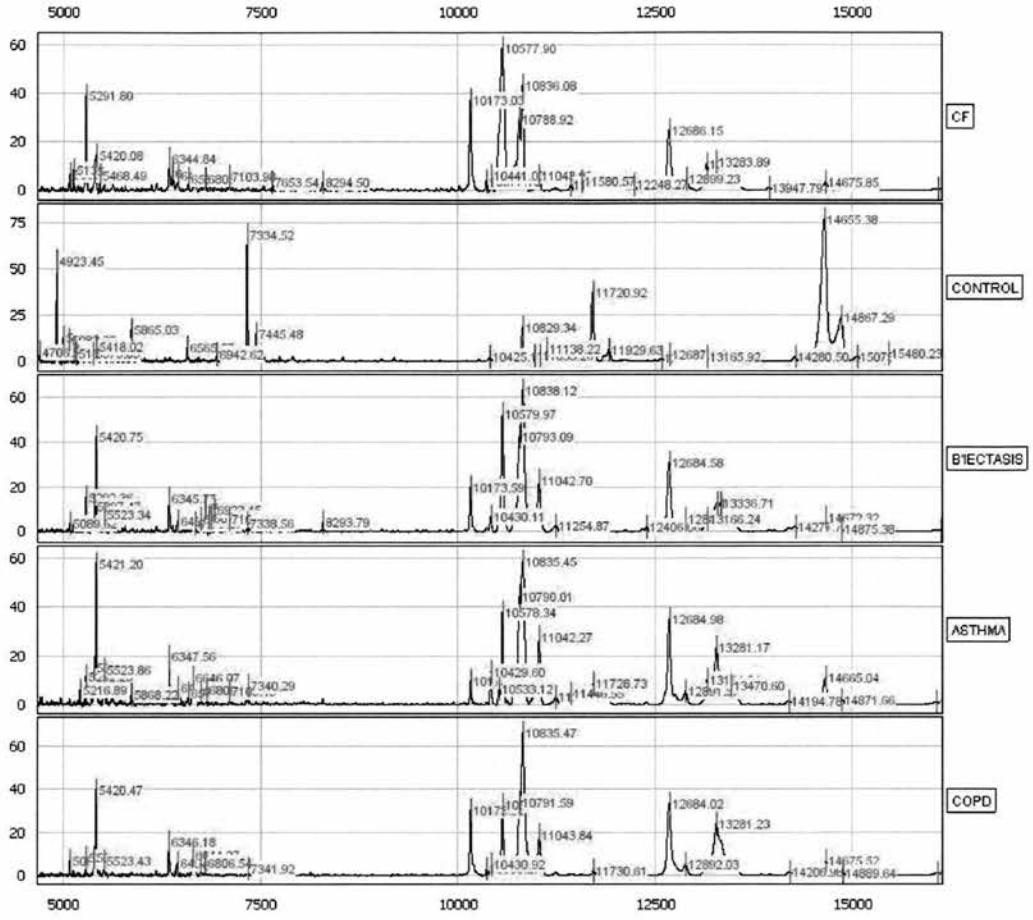


FIGURE 4.1 MASS SPECTRA FROM INDIVIDUAL SUBJECTS

CF, Bronchiectasis, COPD, Asthma and control spectra are shown. Sputum samples were processed and then applied to IMAC Ni ProteinChips. SELDI TOF MS was performed and individual spectra for each subject generated. Protein profiles are demonstrated for the mass range 5 – 20 kDa, clear differences in peak profiles are visible between subjects.

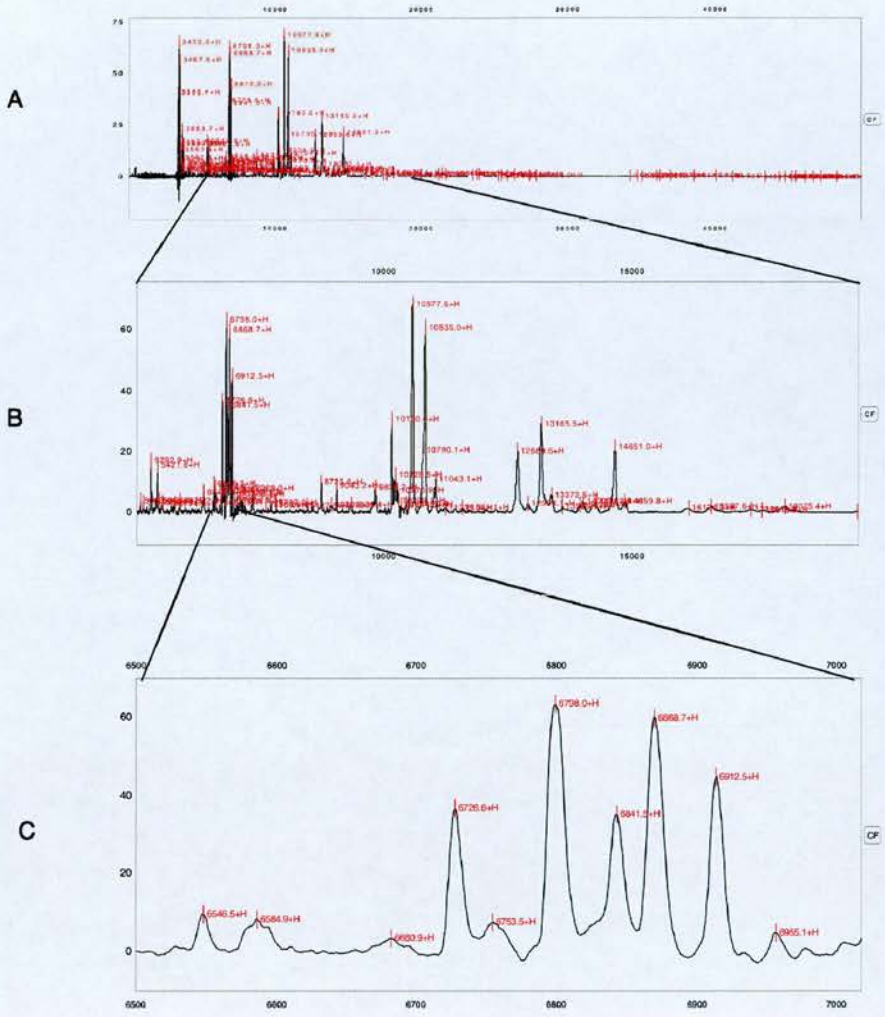


FIGURE 4.2 MAGNIFIED SPECTRA DISPLAY ABUNDANCE OF PEAKS

Spectra from an individual with CF on the CM10 pH4 (cationic exchange) surface shown at different magnifications demonstrating the abundance of peaks in an individual sample. Panel A demonstrates the entire spectrum (3000-50000 Da); each label (most of which cannot be discerned individually at this magnification) represents a peak with signal to noise ratio of >3. Panel B demonstrates some magnification to cover the spectrum from 5000 to 20000 Da and again most of the individual labels cannot be seen clearly at this magnification. Panel C demonstrates magnified spectrum from 6500 to 7000 Da and at this level the individual peaks can be easily discerned.

A

Cation Exchange	Number of Clustering Peaks	Number of Differentiating Peaks p<0.05	**Number P<0.05 Corrected*
Asthma	47	13	*2*
Bronchiecatsis	43	39	*29*
Cystic Fibrosis	44	36	*28*
COPD	38	12	*2*

B

Anion Exchange	Number of Clustering Peaks	Number of Differentiating Peaks p<0.05	**Number P<0.05 Corrected*
Asthma	47	26	*13*
Bronchiectasis	45	39	*34*
Cystic Fibrosis	38	35	*28*
COPD	46	25	*14*

C

IMAC Nickel	Number of Clustering Peaks	Number of Differentiating Peaks p<0.05	**Number P<0.05 Corrected*
Asthma	35	9	*1*
Bronchiectasis	49	40	*48*
Cystic Fibrosis	55	51	*46*
COPD	45	25	*5*

Table 4.2 No of Protein Peaks Differentiating Disease Groups From Control

A, B and C represent the number of protein peaks differentiating disease groups from control subjects over the cation exchange, anion exchange and IMAC nickel surface respectively. *P values were corrected with Bonferoni. Number of clustering peaks refers to the number of peaks compared between each group and control. The majority of differentiating peaks were in the CF and bronchiectasis groups.

4.3.4 Differentiation between Groups Using Principal Component Analysis

Principal component analysis was performed by an automated algorithm on Ciphergen Express Software (Ciphergen, Freemont, Ca, USA). This allows one to then perform 3 dimensional principal component plots demonstrating that CF and Bronchiectasis group together separately from asthma, controls and COPD on all 3 surfaces. This is demonstrated in figure 4.3 for three chip surfaces covering the complete data set.

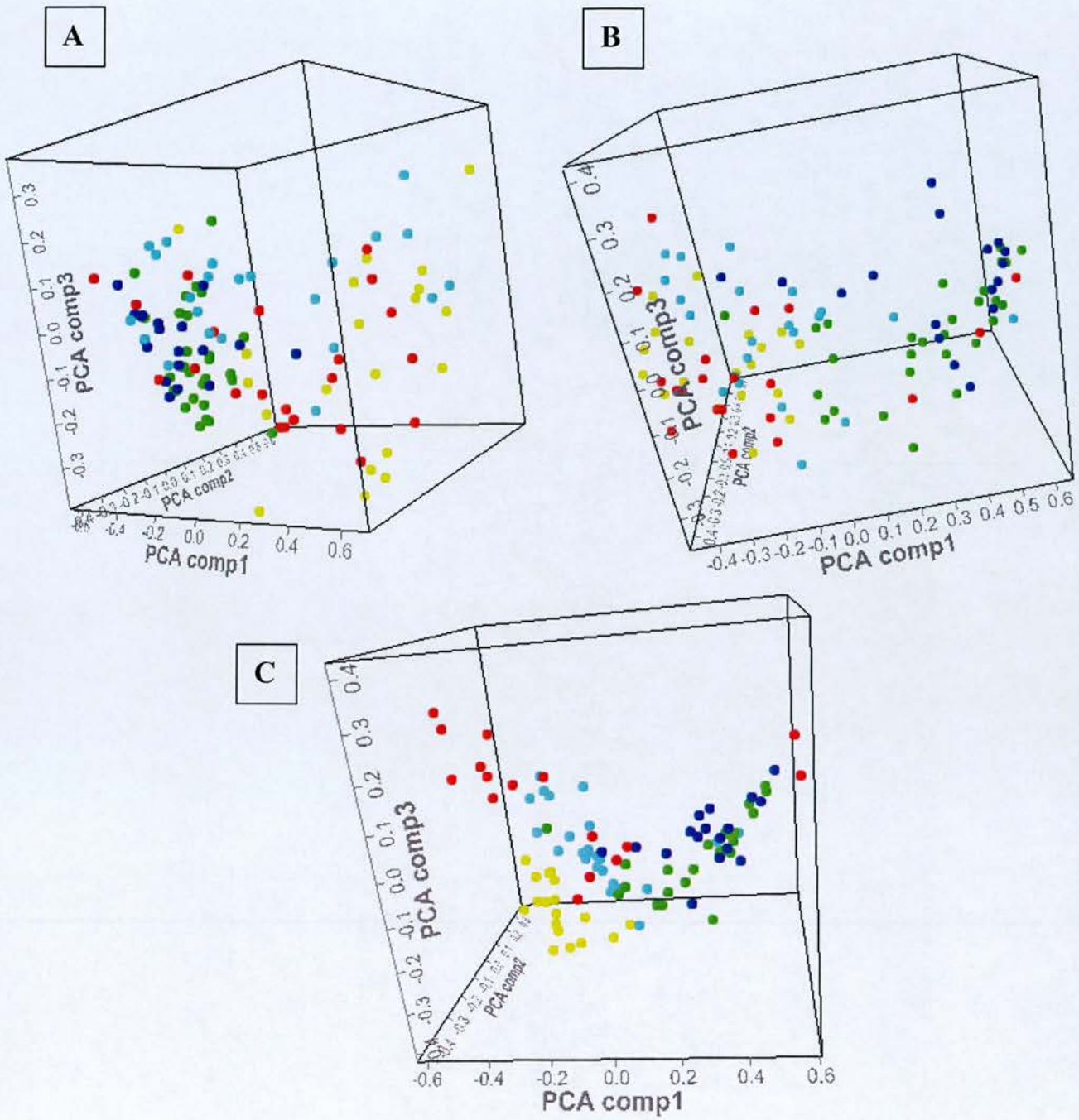


FIGURE 4.3 PRINCIPAL COMPONENT ANALYSIS OF ALL SUBJECTS ACROSS 3 CHIP SURFACES.
Each plot represents the results of analysis from a full experiment and includes all subjects utilised in the study. A: IMAC Nickel, B: CM10, C: Q10. Each Coloured dot represents an individual subject. Green dots are CF, blue bronchiectasis, turquoise control, red asthma and yellow COPD. Principal components were calculated by an automated correlation matrix. On all surfaces CF and Bronchiectasis patients co-segregate from other groups, most impressively so on the IMAC Ni (A).

4.3.5 Recognition of the Most Statistically Significant Biomarkers CF Patients Vs. Controls by Statistical Ranking

By ranking all biomarkers over the 3 chip surfaces a list of the most differential peaks on SELDI TOF is generated. This ranking relies on the statistical significance of the biomarkers rather than the abundance. The 3 most significant markers for CF vs. control were 10531, 10576 and 12244 Da respectively on the IMAC ni surface. A similar peak (to 10576) on the CM10 surface was seen at 10582 Da and represents the same protein. This has now been identified as Calgranulin A.

Molecular Weight Da	Surface	P value	*Direction of Change in CF*
10576*	IMAC Ni	0.0000004	*Increased*
10531	IMAC Ni	0.0000004	*Increased*
12244	IMAC Ni	0.0000004	*Increased*
10786.55	IMAC Ni	0.0000006	*Increased*
12893.18	IMAC Ni	0.0000006	*Increased*
10582.34*	CM 10	0.0000006	*Increased*
12681.95*	IMAC Ni	0.0000007	*Increased*
12697.67*	CM 10	0.0000008	*Increased*
13372.49	CM 10	0.0000010	*Increased*
24003.81	IMAC Ni	0.0000011	*Increased*
7933.153	CM 10	0.0000012	*Decreased*
7948.071	CM 10	0.0000012	*Decreased*
10173.6	IMAC Ni	0.0000012	*Increased*
13173.33	CM 10	0.0000015	*Increased*
4923.537	IMAC Ni	0.0000016	*Decreased*
15485.36	IMAC Ni	0.0000016	*Decreased*
34583.18	IMAC Ni	0.0000016	*Increased*
8844.436	Q 10	0.0000018	*Increased*
11046.58	CM 10	0.0000020	*Increased*
11736.38	*CM 10*	*0.0000020*	*Decreased*

Table 4.3 Markers Differentiating CF from Control

The top twenty sputum markers ranked by statistical significance for CF vs. control are given indicating the chip surface on which they were identified and their molecular weights. Proteins with (*) are the same peak with a similar molecular weight appearing on 2 surfaces and represent the same protein, based on known identification and known protein properties.

4.3.6 The Most Significant Biomarker Differentiating CF From Other Groups

Biomarker 10576 Da on the Nickel surface also differentiated CF from control, asthma and COPD but not from bronchiectasis. This biomarker was identified as calgranulin A following the identification steps described in material and methods and the relative expression on SELDI TOF MS is demonstrated in figure 4.4. The differential presence of calgranulin A was confirmed with western blotting in pooled sputum samples from CF and control patients (figure 4.4). A peak at 10832 was also identified as calgranulin A by mass finger printing following purification on SDS PAGE (see later sections). The relative expression of calgranulin A 10832 across all groups is shown in figure 4.5.

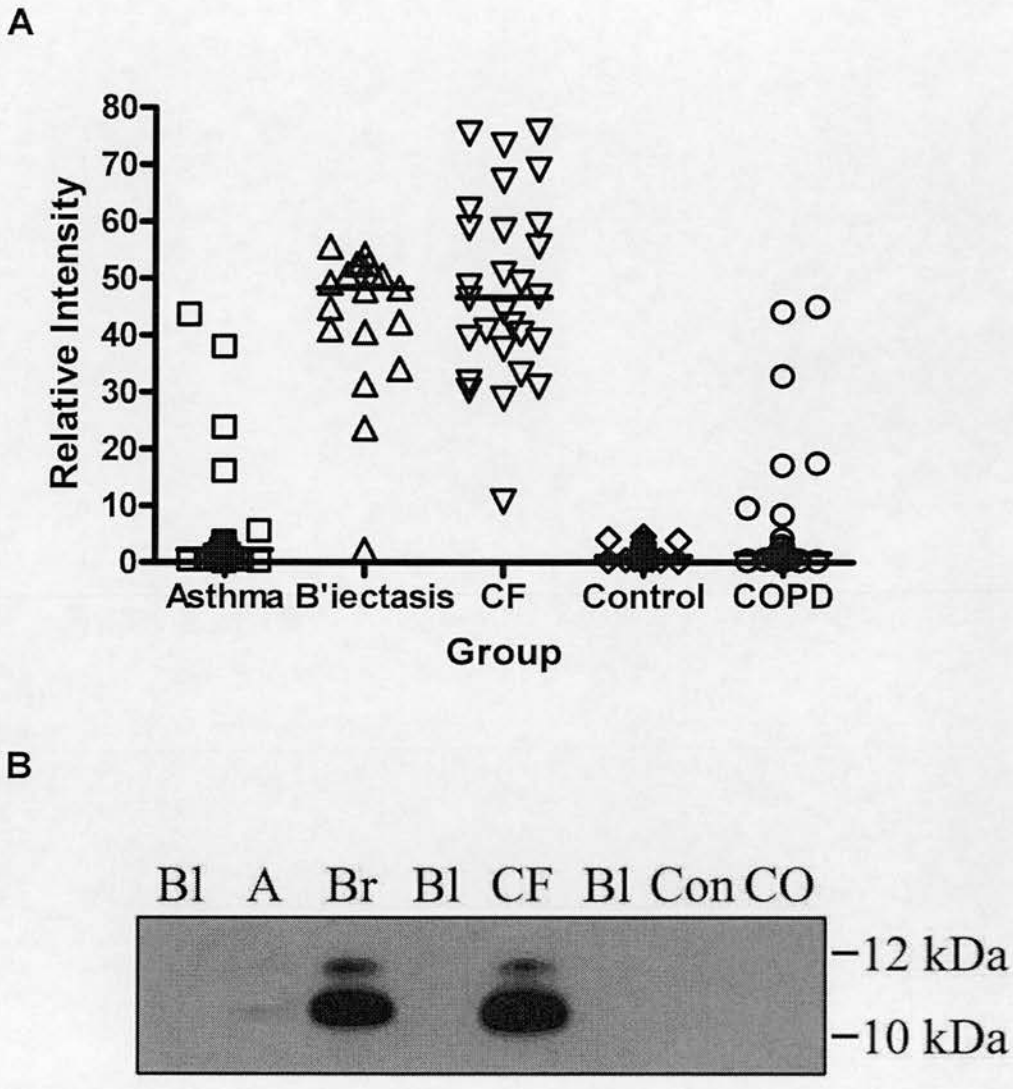


FIGURE 4.4 CALGRANULIN A (10576DA) DIFFERENTIATES CF AND BRONCHIECTASIS FROM OTHER GROUPS

A. Biomarker at 10576 Da (calgranulin A) on IMAC Ni Surface Differentiates CF and bronchiectasis from control and other disease groups ($p<0.001$). Bars represent median.

B. This confirmed by western blot in pooled samples of each group (lanes are labelled as Bl= blank; A= asthma; Br= bronchiectasis; CF= cystic fibrosis; Con= control; CO= COPD). When blotted in sputum calgranulin A runs as doublet with predominance of lower molecular weight isoform. Blot representative of three separate experiments. (Western blot utilised mouse anti-human monoclonal antibody at 1/1000 concentration and rabbit anti-mouse conjugated secondary antibody at 1/1000, 1 minute exposure).

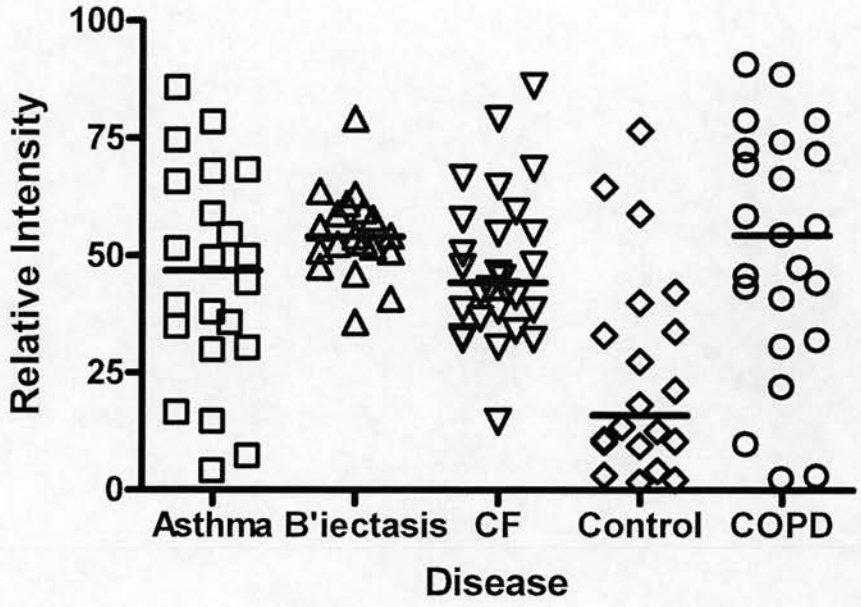
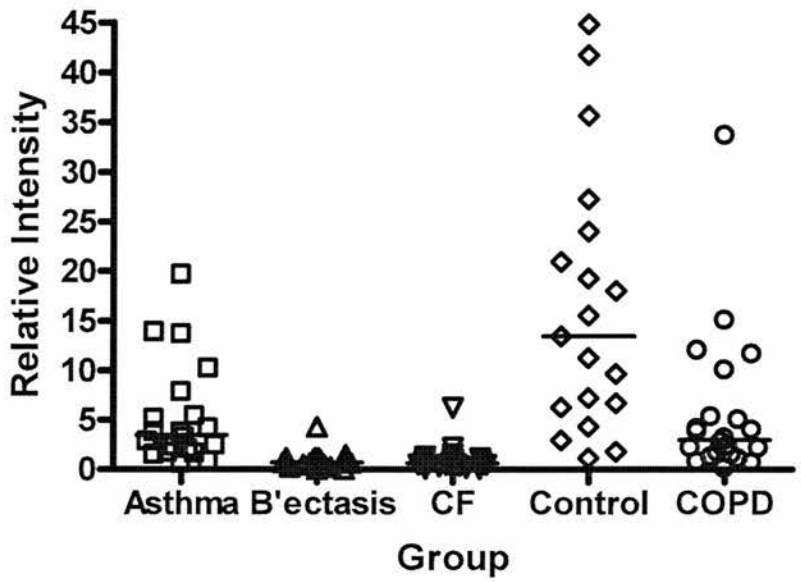


FIGURE 4.5 NATIVE MOLECULAR WEIGHT CALGRANULIN A IS INCREASED IN ALL DISEASE GROUPS
Biomarker 10832 (Calgranulin A) on IMAC Ni surface is elevated in all disease groups vs. control (CF and Bronchiectasis $p<0.001$; Asthma and COPD $p<0.01$). There is no differentiation between disease groups as with 10576 Calgranulin A. Bars represent median.

4.3.7 Down Regulated Protein Biomarkers

The majority of differential biomarkers identified in the CF and bronchiectatic groups were up regulated when compared to the control samples. A limited number were however down regulated. The most significantly decreased protein in CF was at 7.9 kDa on the weak cation exchange and was later identified as clara cell secretory protein, this identification being confirmed by western blot. The relative expression of this biomarker is demonstrated in figure 4.6.

A



B

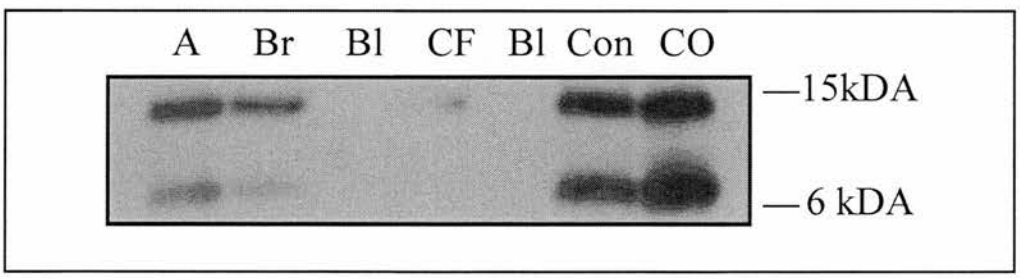


FIGURE 4.6 CLARA CELL SECRETORY PROTEIN IS DECREASED IN CF

Clara cell secretory protein (CCSP), M.W. 7.9kDa, was in lower abundance for all disease groups compared to controls (CF and bronchiectasis vs. control $p<0.0001$; asthma and COPD vs. control $p<0.01$), on CM10 surface. Bars represent median. **B.** This confirmed by western blot in pooled samples of each group (lanes are labelled as Bl= blank; A= asthma; Br= bronchiectasis; CF= cystic fibrosis; Con= control; CO= COPD). CCSP runs as doublet with 7.5 kDa protein and 15kDa dimer (Blot utilised rabbit ant-human polyclonal antibody to CCSP at 1/1000 and pig anti-rabbit conjugated antibody 1/1000, 5 minute exposure). There is strong staining in control and COPD and weaker staining in bronchiectasis and asthma. Blot representative of two separate experiments.

4.3.8 Proteins Differentiating Between Disease Groups

CF and bronchiectasis demonstrated similar biomarker profiles to each other, albeit the abundance of protein in the CF group was greater. Only 5 proteins differentiated CF from bronchiectasis at $p<0.05$ following correction for multiple comparisons. Two of these proteins were observed on the IMACNi surface and 3 on Q10. These results are summarised in table 4.3.3. Further analysis revealed a number of peaks differentiating CF from the disease groups of Asthma and COPD. 29 and 30 proteins differentiated CF from asthma and COPD respectively on the IMAC Nickel surface at $p<0.05$; 27 and 24 on the CM10 surface; and 16 and 14 on the Q10 surface. The 20 most significant proteins in for each disease group on each surface are listed in table 4.3.4. Only 1 peak differentiated between asthma and COPD over the three surfaces and this is displayed in figure 4.7.

Molecular Weight	Surface	p value	*Direction of Change in CF*
17410.19	Q 10	0.0120	*Increased*
5146.118	IMAC Ni	0.0139	*Increased*
5421.827	IMAC Ni	0.0288	*Decreased*
14241.45	Q 10	0.0352	*Increased*
6924.865	Q 10	0.0387	*Decreased*

Table 4.4 Proteins Differentiating CF and Bronchiectasis

Only 5 protein peaks are found to differentiate CF from bronchiectasis following correction for multiple comparisons. The most statistically significant differential protein was discovered on the anionic exchange at a molecular weight of 17410 Da. This protein remains unidentified.

A

Molecular Weight Da	Surface	P value	*Direction of Change in CF*
10532	IMAC Nickel	0.0000004	*Increased*
10576	IMAC Nickel	0.0000009	*Increased*
15857	CM 10	0.0000020	*Increased*
10583	CM 10	0.0000030	*Increased*
6396	CM 10	0.0000038	*Increased*
7947	CM 10	0.0000043	*Decreased*
21139	IMAC Nickel	0.0000056	*Increased*
5295	IMAC Nickel	0.0000063	*Increased*
12893	IMAC Nickel	0.0000063	*Increased*
21380	IMAC Nickel	0.0000063	*Increased*
4923	IMAC Nickel	0.0000086	*Decreased*
6509	CM 10	0.0000112	*Decreased*
10174	IMAC Nickel	0.0000131	*Increased*
23251	IMAC Nickel	0.0000145	*Increased*
4883	CM 10	0.0000159	*Decreased*
14665	IMAC Nickel	0.0000161	*Decreased*
14872	IMAC Nickel	0.0000178	*Increased*
7337	IMAC Nickel	0.0000198	*Increased*
11723	IMAC Nickel	0.0000242	*Increased*
4867	CM 10	0.0000284	Decreased

B

Molecular Weight Da	Surface	P value	*Direction of Change in CF*
10532	IMAC Nickel	0.0000006	*Increased*
4923	IMAC Nickel	0.0000019	*Increased*
10576	IMAC Nickel	0.0000019	*Increased*
10584	CM 10	0.0000036	*Increased*
21139	IMAC Nickel	0.0000043	*Increased*
10981	Q 10	0.0000057	*Increased*
11052	Q 10	0.0000064	*Increased*
10174	IMAC Nickel	0.0000072	*Increased*
21381	IMAC Nickel	0.0000080	*Increased*
4843	IMAC Nickel	0.0000121	*Increased*
10167	Q 10	0.0000129	*Increased*
10574	Q 10	0.0000129	*Increased*
23251	IMAC Nickel	0.0000199	*Increased*
5374	IMAC Nickel	0.0000219	*Increased*
6397	CM 10	0.0000239	*Increased*
15485	IMAC Nickel	0.0000242	*Increased*
15397	Q 10	0.0000317	*Increased*
11139	Q 10	0.0000396	*Increased*
4707	IMAC Nickel	0.0000435	*Increased*
5295	IMAC Nickel	0.0000478	Increased

Table 4.5 Protein Peaks Differentiating CF from Asthma and COPD

The twenty most differential peaks for CF vs. Asthma (**A**) and COPD (**B**). Peaks are ranked by statistical significance. There protein peaks differentiating CF from both asthma and COPD are similar, as were the profile of COPD and Asthma subjects.

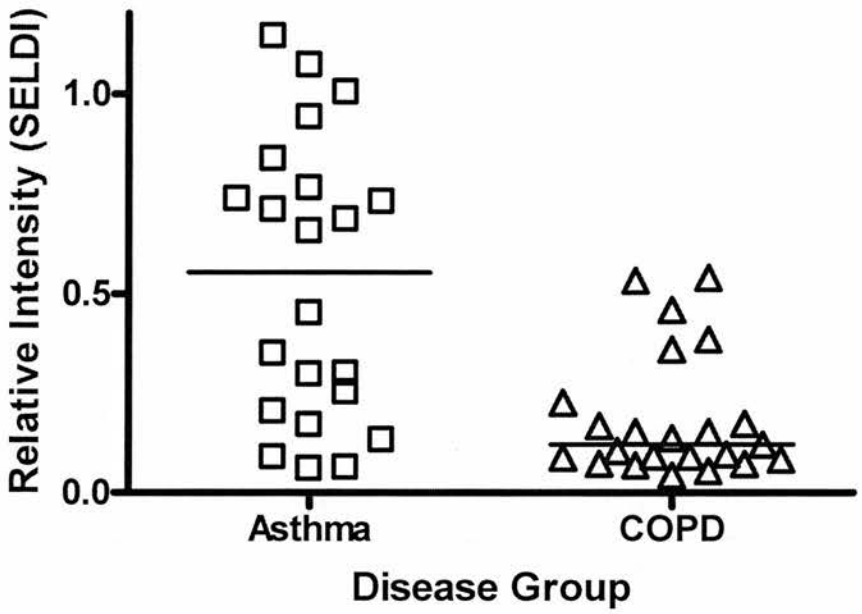


FIGURE 4.7 29KDA PROTEIN DIFFERENTIATES ASTHMA FROM COPD ON SELDI TOF MS
One protein peak separated asthma from COPD patients with SELDI TOF MS ($P<0.05$). This protein had a molecular weight of 29 kDa, and was detected on the IMAC Ni surface. This biomarker has remained unidentified.

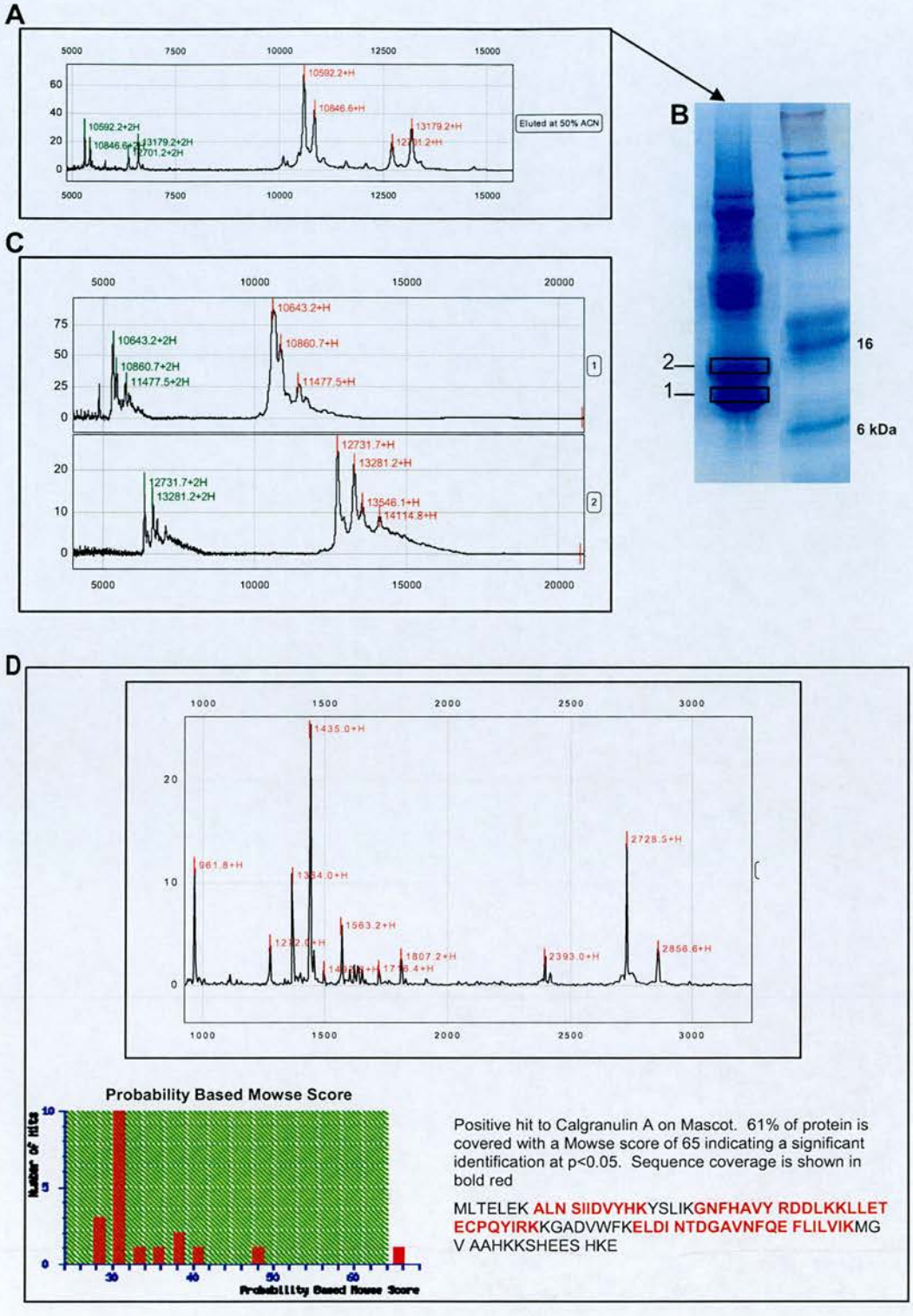
4.3.9 Correlation of Calgranulin A and B with $FEV_1$and Sputum Neutrophil%

Identification of key biomarkers is covered in the following sections. I wished to assess the relationship of our top identified biomarkers with other markers of disease activity and airways inflammation in the CF patients in order to assess the clinical relevance of these markers so as to understand their potential usefulness for development into future assays. Calgranulin A and Calgranulin B demonstrated no significant correlation with FEV_1% predicted and differential sputum neutrophil count. Clara cell secretory protein demonstrated a positive correlation with FEV_1% predicted (Spearman rank 0.43, $p<0.05$) but no relationship to differential neutrophil count.

4.3.10 Proteins Identified From Disease Groups

This chapter has focussed on the development of markers of CF lung disease. Thus an attempt was made to identify biomarkers with relevance to CF lung disease. The proteins listed in table 4 were identified from pooled samples of sputum from the CF and control groups and were confirmed by antibody-based techniques where possible. As a number of the differential protein peaks were in the sub 7 kDa range, these have proved difficult to identify due to the technical problems of resolving peptides in this mass range on polyacrylamide gel. Figure 4.8 demonstrates the identification of calgranulin A and B from sputum with pre-fractionation, resolution on gel, passive elution, trypsin digestion and peptide mass fingerprinting using the SELDI TOF MS platform. Data is also demonstrated for confirmatory MS/MS in figure 4.9. Figure 4.10 demonstrates the identification clara cell secretory protein (CCSP, uteroglobin) using MSMS (Q-TOF), as peptide mass fingerprinting was

inconclusive for this protein. A further number of proteins were identified from MSMS (Q-TOF) without prior mass fingerprinting and these are listed in table 4.3.3. The positive identifications of calgranulin A and clara cell secretory protein were confirmed with western blotting using specific commercial antibodies and are demonstrated previously in the results section. Quantitative immunosassays for these proteins and other known markers of CF will be discussed in the following chapters.



Positive hit to Calgranulin A on Mascot. 61% of protein is covered with a Mowse score of 65 indicating a significant identification at $p<0.05$. Sequence coverage is shown in bold red

MLTELEK **ALN SIIDVYHK**YSLIK**GNFHAVY RDDLKKLLET ECPQYIRK**KGADVWFK**ELDI NTDGAVNFQE FLILVIK**MG V AAHKKSHEES HKE

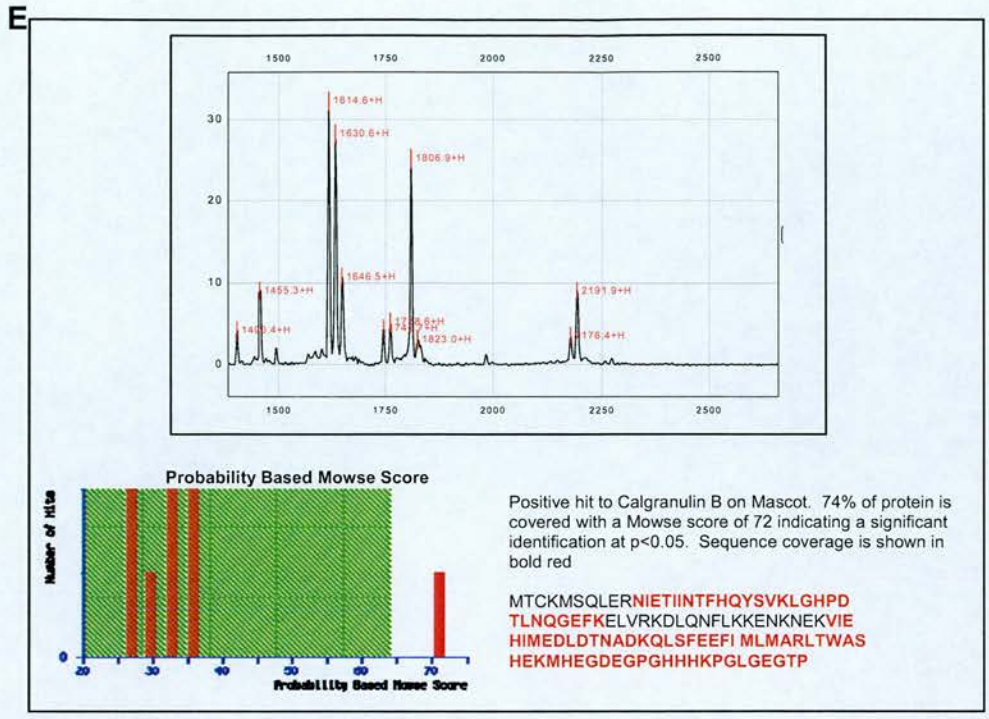
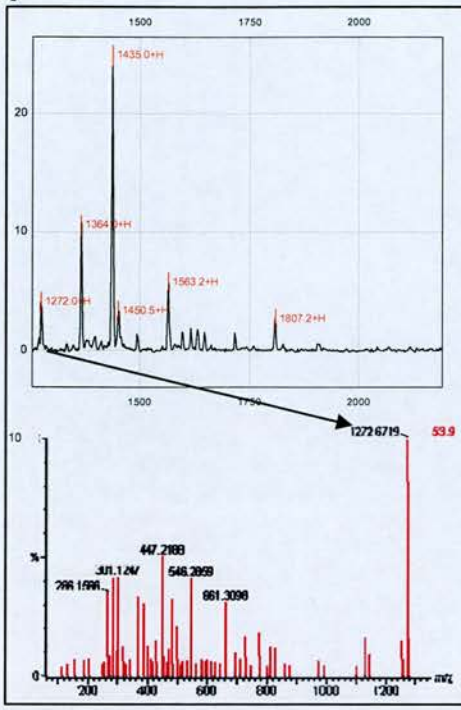
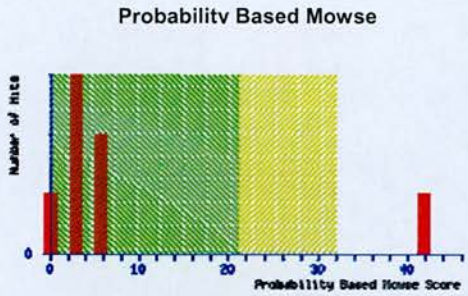


FIGURE 4.8. IDENTIFICATION OF CALGRANULINS A AND B BY PEPTIDE MASS FINGERPRINTING ON SELDI TOF MS PLATFORM.

A: Pooled CF sputum sample was purified reverse phase chromatography and elution with 50% acetonitrile. This was applied to an NP20 ProteinChip and demonstrates dominant peaks in the 10-13 kDa on SELDI TOF MS. The lower molecular weight peaks labelled in green indicate doubly pronated (double charged) protein. **B:** This eluted sample was run on 18% tris/glycine gel, stained with coomassie and the bands cut as shown. **C:** Passive elution of protein from the cut gel bands demonstrates the further purification of peaks around 10576 Da and 13200 Da. **D:** Passive elution from band 1 was digested with trypsin and applied to an NP20 ProteinChip. The resultant peptides were subjected to a Mascot search (Matrix Science Inc, MA, USA) and demonstrated a highly significant hit for calgranulin A with probability based Molecular Weight Score (MOWSE) of 72 (score of >64 indicates significant identification at $p<0.05$). Sequence coverage of 61% by fragments is demonstrated in bold red. **E:** Trypsin digest of passive elution from band 2 processed as in D, demonstrating a highly significant hit for calgranulin B MOWSE score of 72 and coverage of 71%.

A

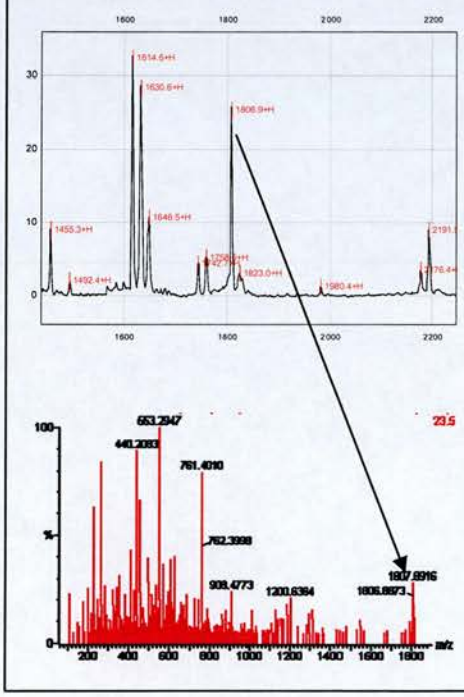


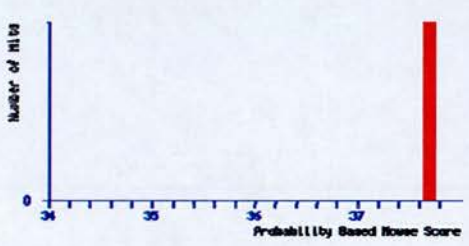


Positive hit to Calgranulin A on Mascot of fragmented peptide (amino acid Sequence) with a Mowse score of 42 indicating a significant identification at $p<0.05$. Sequence coverage is shown in bold red.

MLTELEK**ALNSIIDVYHK**YSLIKGNFHA
VYRDDLKKLLETECPQYIRKKGADVWF
KELDINTDGAVNFQEFLILVIKMGVAAH
KKSHEESHKE

B





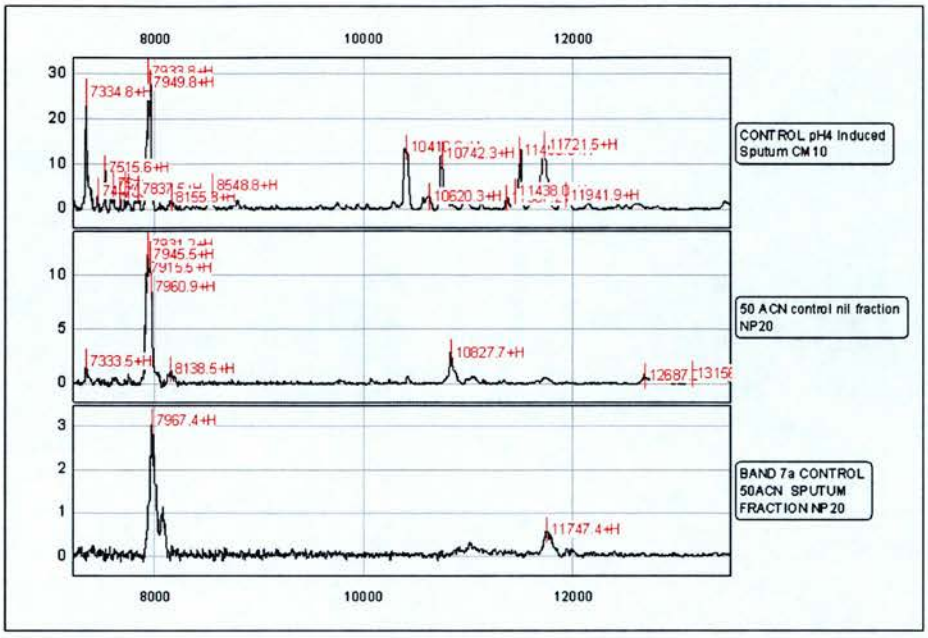
Positive hit to Calgranulin B on Mascot of fragmented peptide (amino acid Sequence) with a Mowse score of 38 indicating a significant identification at $p<0.05$. Sequence coverage is shown in bold red.

MTCKMSQLER**NIETIINTFHQYSVK**LGHP
DTLNQGEFKELVRKDLQNFLKKENKNEKV
IEHIMEDLDTNADKQLSFEEFIMLMARLTW
ASHEKMHEGDEGPGHHHKPGLGEGTP

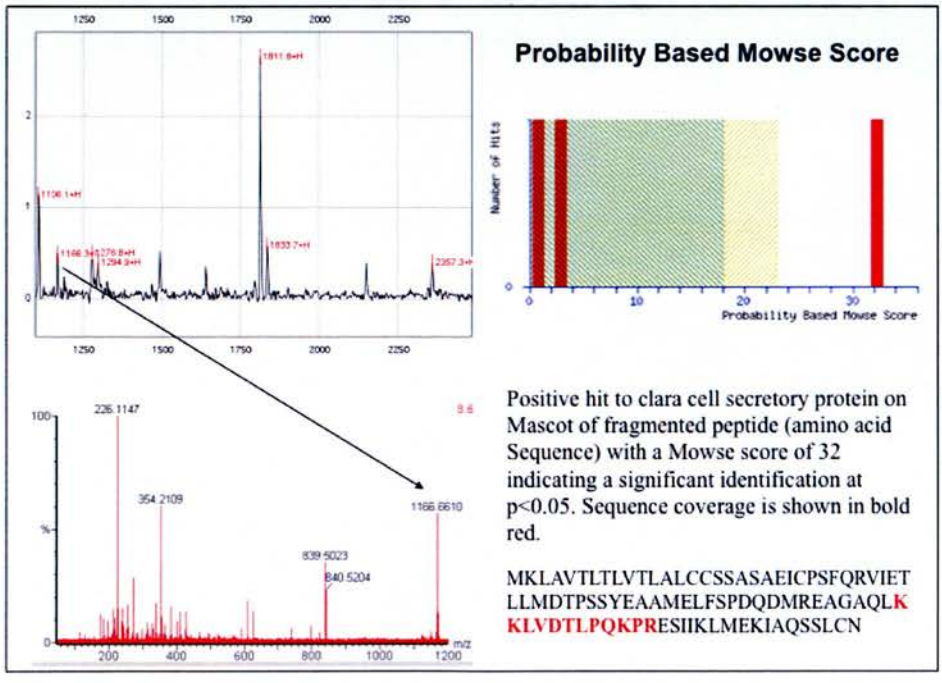
FIGURE 4.9 CONFIRMATION OF CALGRANULIN A AND B WITH MS/MS (Q-TOF)

Proteins were purified as in figure 4.8. Samples were subjected to MSMS (Q-TOF) Individual peptides were selected from trypsin digests spotted on NP20 ProteinChips (seen in figure 4.8, D and E) by a quadropole and subjected to collision-induced dissociation followed by TOF MS. **A:** Peptide at 1272 Da was dissociated and sequenced as calgranulin A with significant MOWSE score of 42 ($p<0.05$). **B:** Peptide at 1806 Da was selected and following dissociation was sequenced as calgranulin B with significant MOWSE score of 38 ($p<0.05$). Experiments were performed on Applied Biosystems QSTAR tandem MSMS (Q-TOF), with ProteinChip interface by Nathan Harris (Ciphergen, UK).

A



B



Positive hit to clara cell secretory protein on Mascot of fragmented peptide (amino acid Sequence) with a Mowse score of 32 indicating a significant identification at $p<0.05$. Sequence coverage is shown in bold red.

MKLAVTLTLVTLALCCSSASAEICPSFQRVIET
LLMDTPSSYEAAMELFSPDQDMREAGAQLK
KLVDTLPQKPRESIIKLMEKIAQSSLCN

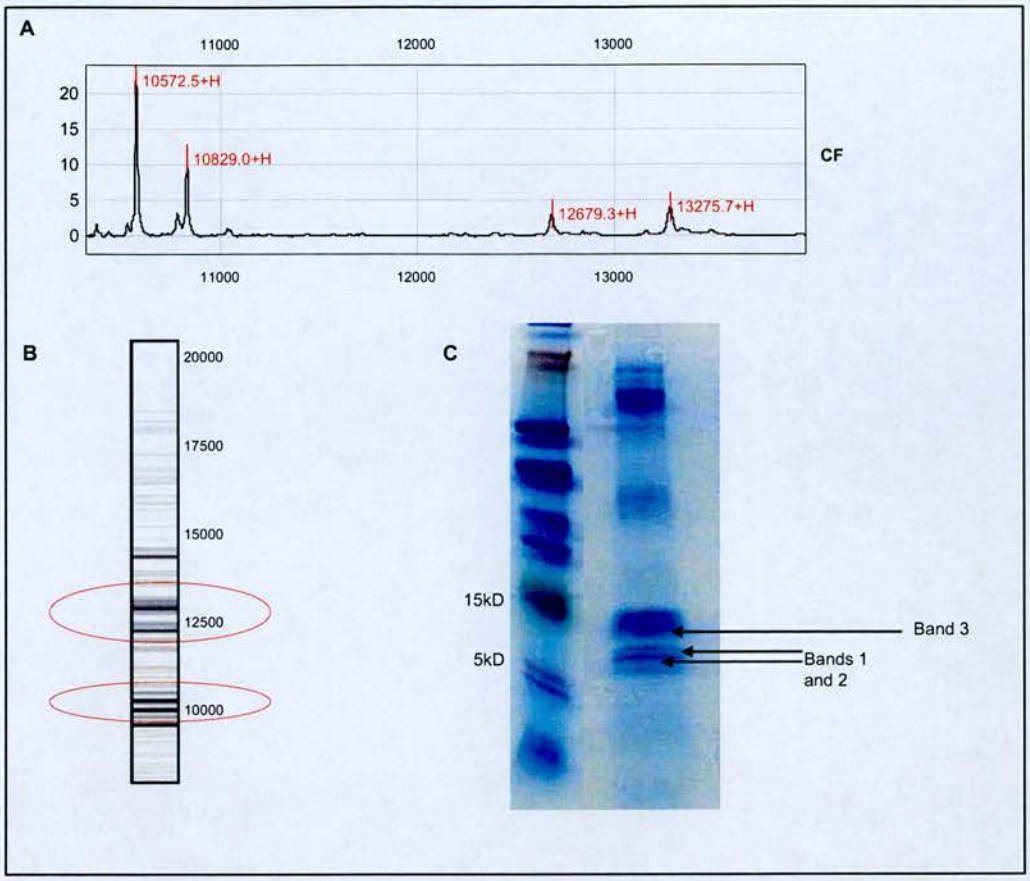
FIGURE 4.10 THE IDENTIFICATION OF CLARA CELL SECRETORY PROTEIN

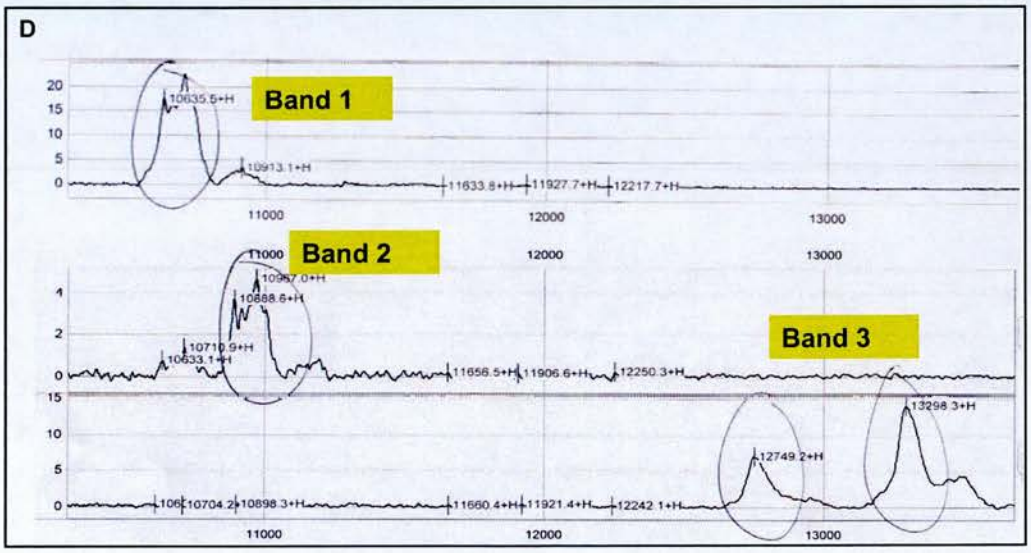
A. Reverse phase chromatography and gel electrophoresis was performed as in figure 4.8. Panel A demonstrates the original spectrum from a control subject, the reverse phase purification and the eluted protein from gel band leading to a successively purified protein sample. B. Following purification the protein at 7900 Da was digested with trypsin and mass spectrometry performed on the resultant peptide fragments. Peptide mass fingerprinting did not return a positive identification and therefore Q-TOF analysis was performed on peptide fragment at 1166.3 Da from and amino acid sequence derived with good peptide coverage for clara cell secretory protein (CCSP, uteroglobin) with a MOWSE score of 32 ($p<0.05$). CCSP was further confirmed in sputum samples of control subjects by western blotting (see figure 4.6 panel B).

Protein	Predicted Molecular Weight	Accession Number	Corresponding SELDI Peak (approx)	Confirmed By PMF	Confirmed by MS/MS	Confirmed By Antibody	Direction of Change in CF
Calgranulin A	10834	P05109	10832, 10576	yes	yes	yes	*Increased*
Calgranulin B	12960	P06702	12960, 13200	yes	yes	yes	*Increased*
Calgranulin C	10100	P80511	10100	no	yes	no	*Increased*
Clara Cell Secretory Protein	7900	P11684	7900	no	yes	yes	*Decreased*
Proline Rich Salivary Peptide	8188	P02814	8119	no	yes	no	*Decreased*
Lysosyme C	16537	P61626	14600	yes	yes	no	*No Change*
Cystatin S	16204	P01036	16079	no	yes	no	*No Change*
Haemoglobin Alpha	15117	P69905	15080	no	yes	no	*No Change*

Table 4.6 Protein identifications confirmed from present study.

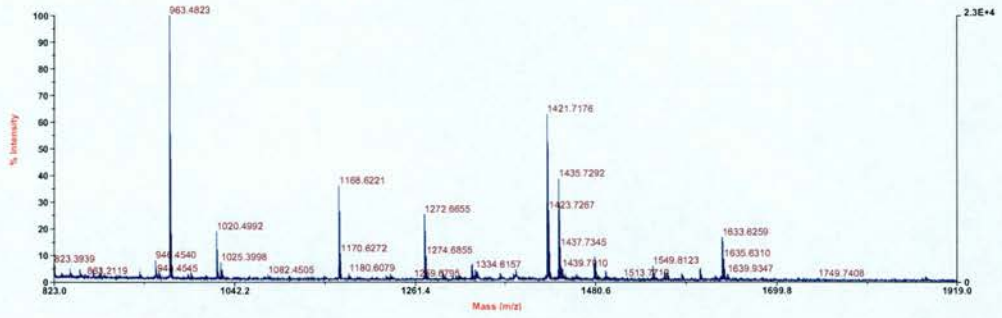
Predicted molecular weight and accession number are displayed for identified proteins. Proteins were identified by trypsin digest and peptide mass fingerprinting (PMF) and well as tandem (Q-TOF) MS/MS. When available, antibodies were employed to confirm protein identification by Western Blot. Molecular weight refers to the theoretical molecular weight of each protein as derived from sequence. Corresponding SELDI peak refers to the protein peak seen on SELDI TOF MS analysis of sputum fluid phase (differences in molecular weight may represent post translational modifications). Direction of change in CF represents direction of change compared to control samples.


A
11000
12000
13000
10572.5+H
10829.0+H
12679.3+H
13275.7+H
CF
20
15
10
5
0
B
20000
17500
15000
12500
10000
C
15kD
5kD
Band 3
Bands 1
and 2


D
Band 1
Band 2
Band 3
10635.5+H
10913.1+H
11633.8+H
11927.7+H
12217.7+H
10957.0+H
10688.6+H
10710.9+H
10633.1+H
11656.5+H
11906.6+H
12250.3+H
13298.3+H
12749.2+H
10704.2
10898.3+H
11660.4+H
11921.4+H
12242.1+H
11000
12000
13000

E

Band 1
Calgranulin A

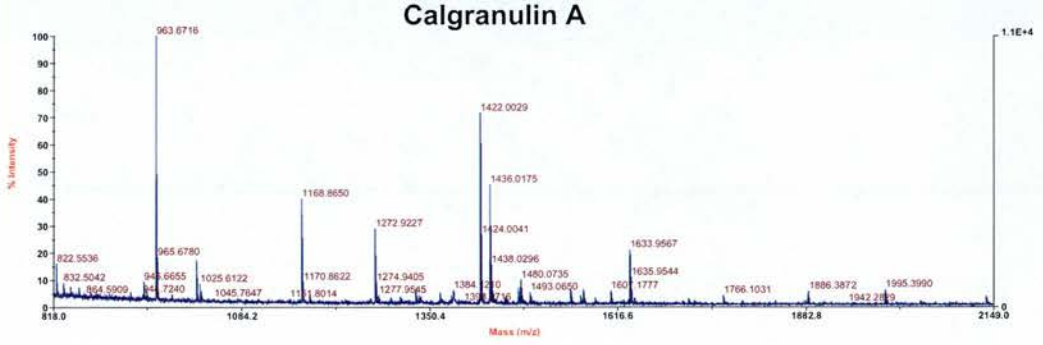


MLTELEKALNSIIDVYHKYSLIKGNFHAVYRDDLKKLLETECPQYIRKKGADV WFKELDINTDGAVNFQEFLILVIKMGVAAHKKSHEESHKE

Peptide Fragments cover 60% of the protein sequence for Calgranulin A. Proving that lower molecular weight peak is calgranulin A. Mowse score for this mass fingerprint is 18,100 indicating high certainty of identification.

F

Band 2
Calgranulin A



MLTELEKALNSIIDVYHKYSLIKGNFHAVYRDDLKKLLETECPQYIRKKG ADVWFKELDINTDGAVNFQEFLILVIKMGVAAHKK**SHEESHK**E

Peptide Fragments cover 67% of the protein sequence for Calgranulin A. Proving that higher molecular weight peak is calgranulin A. Mowse score for this mass fingerprint is 263,000 indicating high certainty of identification.

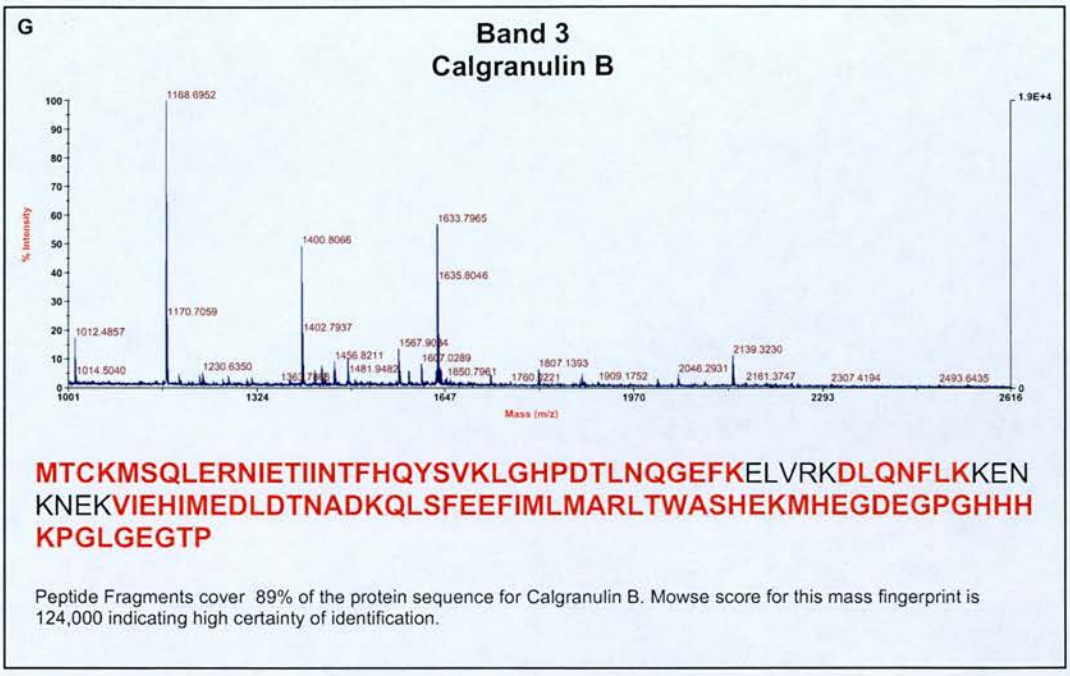


FIGURE 4.11 FURTHER CHARACTERISATION OF CALGRANULIN A AND B

A: the appearance of peaks in an individual sample previously identified as Calgranulin A and B on CM10 pH4 surface. **B:** a simulated gel view of SELDI TOF MS spectra from A showing the close proximity of two bands around 10.6 kDa and 10.8 kDa as well as further bands at 12-13 kDa. **C:** Pooled sputum samples from CF patients were resolved by 18% tris-glycine PAGE demonstrating a similar pattern to the simulated gel. Gel bands were cut as shown and half of the material used for passive elution, the other half subjected to in gel trypsin digestion. **D:** Passive elution demonstrates bands 1 and 2 being of different molecular weights at approximately 10.6 and 10.9 kDa. Band 3 is a mixture of molecular weights of 12.7 and 13.2 kda. Panels **E**, **F** and **G** demonstrate the peptide mass fingerprints on MALDI TOF and sequence coverage of fragments following trypsin digest and show that both the peak at 10.6 and 10.9 kDa represent calgranulin A and that the higher molecular weight band, a mixture of 12.7 and 13.2 kDa material, is Calgranulin B. Database searches were performed on MS FIT (University of California, San Francisco, USA), a free online peptide search engine. The MOWSE score from MS FIT gives is calculated differently than Mascot with a score of greater than 10000 suggesting a positive identity.

4.4 Discussion

4.4.1 General Findings

The use of SELDI-TOF mass spectrometry has allowed us to generate sputum mass spectral profiles for several diseases and then compare these between groups. There are large numbers of potential protein biomarkers that differentiate CF from healthy subjects and other disease groups. Several of these candidate biomarkers have been identified, including calgranulin A and B and clara cell secretory protein, which were among the top twenty most statistically significant proteins to differentiate CF from control subjects and also differentiated CF from Asthma and COPD (when using Calgranulin A 10576 marker). Thus I have demonstrated the potential of SELDI TOF MS to screen sputum samples for biomarkers and then identified these markers for further characterisation and functional testing by other assays as discussed in further chapters.

4.4.2 SELDI TOF MS as A Screening Tool in Sputum

I chose to use SELDI TOF MS as our primary method for biomarker discovery in this study. SELDI TOF MS provides a rapid high throughput method of proteomics, and has been successfully utilised in a range of diseases and body fluids to identify biomarkers or signature protein profiles(211-219). The application of SELDI TOF to respiratory secretions has been limited so far to studies of bronchoalveolar fluid(223, 224) with two further studies concentrating on CF(174, 175), although other proteomics based biomarker discovery methods have been utilised in CF sputum with success(196, 208, 225). By choosing a mass spectrometry based strategy I aimed to remove any pre-selection bias from the study. A major draw back of using

a mass spectrometry based technology is that it effectively limits the range of proteins that may be discovered as the resolution of mass spectrometry is probably best in the mass range of 1000 to 50000 Da, and thus potentially misses important higher molecular weight proteins. Indeed in sputum I demonstrated that the majority of peaks discovered using the above experimental protocols lay in the 4 to 20 kDa range, consistent with a previous study in CF BAL(174). Nevertheless I have demonstrated large numbers of differential peaks in this mass range and have demonstrated the identification of biologically significant proteins in sputum.

SELDI-TOF MS has been used as a high throughput screening platform for biomarker discovery rather than a primary diagnostic platform. This avoids many of the criticisms levelled at the interpretation of multivariate bioinformatics data in some previous SELDI TOF MS studies. Separation of patient and control groups is possible using principal component analysis of our mass spectral data, but the identification of key biomarkers is more likely to yield assays that ultimately have greater application in the clinic, a view shared by others in the field (222). As mass spectrometry is a specialised technique it is much more likely that a biomarker will be accepted as a clinical test if it is readily measurable, for example by ELISA.

4.4.3 Sputum as an Appropriate Body Fluid

I utilised the fluid phase of sputum as a non-invasive means of assessing the airways. Previous work has demonstrated a correlation of induced sputum with bronchial washings but not BAL (251), and therefore suggests that it reflects the pathophysiology of more central airways(250, 251). Induced sputum has previously

been demonstrated useful sampling method in CF lung disease(102, 183, 194, 195, 197-199, 208, 250, 251, 255, 257-260). Proteomic assessment of sputum is a valid means of assessing airways disease, the caveat being that it may be underestimate the presence of biomarkers derived from the distal airway, however this may be highly relevant to CF where disease is prominent in the bronchial rather than alveolar space. Sputum also offers a non-invasive means of airways sampling in comparison to the methods needed to sample lower airways fluid such as BAL.

A further consideration in this data set is the absence of paired serum samples in the disease and control groups. Serum sampling would have enabled an assessment of whether significant biomarkers were a result of local production and/or release in the lung or due leak of systemically produced/released proteins into the airway. Future proteomic studies of sputum should consider this. Nevertheless I have demonstrated the presence of biologically relevant proteins in the sputum of CF patients compared to control subjects and other inflammatory lung diseases. The potential clinical and functional relevance of these markers will be considered in future chapters.

4.4.4 Differentiating Protein Peaks

The largest numbers of differentiating protein peaks between disease and control were found in CF and bronchiectasis. This may suggest greater numbers of inflammatory and disease related proteins in these groups or alternatively that the samples obtained from these groups had greater abundance of protein. Sputum samples obtained from CF and bronchiectatic subjects were of greater volume and

better quality, subjectively. However, mucus plugs were used for analysis and processed with equal ratios of buffer as previously described and recommended by a recent ERS working group(207, 261). Moreover to ensure samples were comparable when measured on SELDI TOF MS, supernatants were adjusted to a total protein concentration of 1mg/ml before applying to chip surfaces. Also, mass spectral data were normalised to total ion current prior to data analysis, thus minimising effects of possible variation in total protein binding to chip surfaces. These steps were implemented in order to ensure that differences in protein profiles between groups did not simply represent differences in the overall abundance of total protein in each sample group. A lower limit cut-off of 4 kDa was selected for analysis of spectra to select biomarkers. This cut off was chosen to reflect the mass deflector setting employed in the experiments, as spectra below 4000 Da would have been less reliable in terms of accuracy and contained more experimental noise. This may bias the analysis to proteins of molecular weight greater than 4 kDa, whilst also potentially ignoring biomarkers of below 4 kDa. Indeed all spectra contained peaks around 3 kDa with the appearance of alpha defensins, which had to be excluded from analysis due to the preset experimental criteria.

Alternatively, I may have overestimated true numbers of differentiating markers as some proteins are detected on more than one chip surface. Also, a proportion of some proteins may be doubly protonated or "double charged" due to interactions between protein on the chip surface and matrix at the time of ionisation, some proteins picking up 2 protons rather than 1. As the time of flight is related to both MW and charge, such proteins will appear "twice", once with half the MW of the

original protein as it flies twice as fast. Finally different peaks may represent cleavage products of the same (higher molecular weight) proteins although this is only discernable once identification of each peak is available.

Irrespective of the potential confounding factors, large numbers of potential biomarkers are demonstrated. From these I have identified a modest number of proteins, including the most differential up-regulated protein for CF vs. control, calgranulin A (in 10576 Da form), and also the most differential down-regulated protein for CF vs. control, clara cell secretory protein. Further proteins were also identified during this process. The failure to identify a greater number of protein peaks is disappointing and demonstrates the difficulties in protein identification using the SELDI TOF MS platform. Indeed a number of the proteins were only identified following the combination of SELDI TOF MS with tandem MSMS (Q-TOF). Further strategies such as shotgun profiling may offer a more realistic approach to identifying a larger number of proteins(225), although such methods are still in developmental stages and will require further validation.

A further consideration whilst identifying possible candidate biomarkers in sputum from cystic fibrosis is that proteins could be eukaryotic as well as prokaryotic. This has not been borne out so far as all of the proteins identified are human in origin. Indeed, to ensure I only identified human proteins that may be appropriate biomarkers I only searched online repositories for human proteins when using peptide mass fingerprinting data.

In spite of finding a large number of peaks differentiating CF and bronchiectasis from control I was surprised not to have identified any of these as known biomarkers of inflammation such as interleukin 8 or neutrophil elastase. SELDI TOF MS was most efficient at demonstrating peaks in the 5-20 kDa range and this may in part explain these findings as the predicted molecular weights of neutrophil elastase is 29.5 kDa(262). Alternatively, the chip surface chemistries and binding conditions may simply have not favoured preferential selection of proteins such as interleukin-8 which has a predicted molecular weight of 8.9 kDa.

4.3.5 Calgranulin A and B: Calprotectin

In all disease groups I identified calgranulin A at higher levels than controls. In the CF and bronchiectasis groups a protein at 10576 Da was also identified as calgranulin A. This proved to be the most discriminating biomarker between CF and all other groups and represents a cleavage product or post-translational modification resulting in a mass shift of 256 Da. In spite of being able to identify both molecular weights as calgranulin A I have been unable to identify the precise reason for this mass shift but the truncated form may represent a loss of amino acids lysine and glutamic acid from the C terminus, a finding recently reported in an alternative data set from CF BAL fluid(175), and supported by the peptide mass fingerprinting data shown in figure 4.7.

Calgranulins have previously been described in the sera of subjects homo- and heterozygous for CF mutations and referred to as CF antigen (263). Calgranulin A may therefore be a sensitive marker of airways inflammation in CF. I also identified

2 further protein peaks as calgranulin B. Calgranulin B showed a similar expression to calgranulin A in terms of differentiating CF and other diseases from control, but also CF from other diseases. Calgranulin A and B combine to form a heterodimer commonly referred to as calprotectin. The likely source of calgranulin A and B is the airway neutrophil, although it is also produced by macrophages and epithelial cells. Previously known as the L1 protein complex, calprotectin accounts for 40-60% of the non-granular protein load in the neutrophil and has a multipotent role in neutrophils including the transport of calcium intracellularly and the chelation of zinc extracellularly(264), as well as being a potent anti-fungal protein. Furthermore its release from neutrophils has been demonstrated during a novel form of cell death described as NETosis where upon cell death a network of DNA strands and proteins is released with a major protein constituent being calprotectin(265).

Surprisingly there was no correlation between FEV_1 and levels of calgranulin A suggesting that calgranulin levels are associated with lung inflammation but not lung function. Furthermore I failed to demonstrate any significant correlation between neutrophil% in sputum and calgranulin A as measured by SELDI TOF. This may in part be explained by the finding that the majority of the CF patients had a profound sputum neutrophilia and thus calgranulin levels may offer a better understanding of levels of inflammation. Calprotectin (calgranulin A and B) has previously been recognized as a biomarker in other inflammatory disorders, such as inflammatory bowel disease and arthritis (103, 266, 267) thus suggesting further roles as a marker of inflammation in a diverse group of diseases. Furthermore, recent experimental evidence suggests that functional knock out of calprotectin in the lung in an

experimental model of pneumonia decreases the level of inflammatory cell influx into the lung thus further suggesting a pro-inflammatory role(268). Further data has also proposed calgranulin A and B as pro-inflammatory mediators via the activation of Toll Like Receptor 4 (TLR-4), which is a key receptor in the promotion of inflammation(269). Thus the presence of calgranulin in the CF lung may be both a marker of inflammation as well as a (potentially) potent mediator of inflammation.

4.4.6 Clara Cell Secretory Protein (CCSP)

A second abundant protein identified showed opposite effects to calgranulin A. Clara cell secretory protein (CCSP) was reduced in all disease groups compared with controls. CCSP is an anti-inflammatory protein mainly expressed in the epithelial cells of the airways. Low serum levels have been reported in asthma(270), and low nasal lavage levels have also been measured in allergic rhinitis(271). CCSP has a number of possible activities including inhibition of phospholipase A2, chelation of calcium and down regulation of IFN-γ, IL-1 and TNF-α. My findings are consistent with previous clinical studies demonstrating low levels of this protein during airways inflammation, and raise the possibility that resolution of inflammatory processes might be monitored by rising levels of CCSP or alternatively that low levels of CCSP in CF may allow uncontrolled inflammatory stimulation in the lung by other mediators as listed above.

4.4.7 Correlation of Calgranulin and CCSP with Lung Function and Sputum Neutrophil%

I demonstrated no correlation between lung function and sputum neutrophil counts with calgranulin A and B. CCSP was correlated to lung function but not to

neutrophil counts. The primary aim of this study was to differentiate between CF and other control groups in terms of protein peak expression. I have effectively demonstrated this, however any further interpretation as to the clinical significance of these findings is difficult without longitudinal studies. Nevertheless many of the proposed sputum biomarkers in publication have been suggested following cross sectional studies(91, 183, 194, 197-199, 255, 256, 272). It is also important to consider that the CF subjects taking part in this study were from an adult cohort and as such had established lung disease as evidenced by their average FEV_1% predicted of 59%, further studies of proteomics in patients with milder disease are therefore indicated, to investigate the relationship between sputum biomarkers and lung function.

4.5 Conclusions

In conclusion I have demonstrated the utility of SELDI-TOF mass spectrometry as a tool for biomarker discovery in induced sputum. I have positively identified proteins of biological significance in the fluid phase of sputum. These proteins will be assessed in the following chapters to assess their usefulness as clinical assays. I demonstrated not only markers that differentiated CF from control, but also CF from asthma and COPD. In the process of the study I was also able to demonstrate differences in protein profile between asthma and COPD with control but very few differences between asthma and COPD, reflecting the similarities of these diseases at a protein level particularly in our cohort of patients (particularly when we consider that asthmatics attending a hospital clinic may have more severe disease and thus have some phenotypic similarities to the COPD patients). Calgranulin A and B as well as CCSP appear to be suitable candidate biomarkers for CF and will be investigated in future chapters.

5.0 Examination Specific Biomarkers in Cystic Fibrosis Using Enzyme Linked Immunosorbent Assays

5.1 Introduction

5.1.1 Background

As demonstrated in the previous chapters sputum from CF subjects contains an abundance of proteins, which have yet to be fully classified. Several studies have been published utilising immunoassays to measure specific proteins in sputum and have been summarised in chapter 1 of this thesis (table 1.2).

The measurement of protein biomarkers with enzyme linked immunosorbent assays (ELISA) allows a truly quantitative test of protein level in sputum whereas mass spectrometry is semi-quantitative (albeit with high sensitivity). The clinical application of any biomarker will be limited by the expertise and equipment required to make the measurement. For example spirometry is readily performed at the bedside and requires relatively little training whereas techniques such as mass spectrometry require sophisticated hardware and technical expertise. Translating the findings from interesting observations with mass spectrometry to clinically useful tests will therefore require the application of techniques that can be utilised across many centres. ELISA is a well-established technique for measuring proteins in body fluids and forms the basis of many standard clinical tests. Therefore I propose that ELISA is a more clinically applicable means of measuring biomarkers discovered by high throughput SELDI TOF screening and allow a quantitative means of measuring novel biomarkers.

5.1.2 Sputum Calprotectin

Calprotectin is the heterodimer of Calgranulin A and B. Chapter 4 demonstrates its discovery in sputum using SELDI TOF mass spectrometry. On mass spectrometry the most prominent differentiating peak between CF and control subjects is Calgranulin A, part of the Calprotecin complex. Furthermore a peak identified as Calgranulin B was also differential for CF vs. control. Calprotectin is a neutrophil protein which accounts for 40 - 60 % of the non-granular neutrophil cytoplasmic protein load(264). Calgranulin A has previously been described as CF antigen(13) and was measured in the serum of CF patients and heterozygote carriers of CF mutations. Calprotectin has also been previously described as neutrophil L1 complex, and is measurable in the serum of patients with a range of respiratory conditions(273). More recently Calprotectin has been utilised as a clinical test in the field of gastroenterology(274). Ulcerative colitis is a neutrophil mediated inflammatory bowel disease and high levels of calprotectin have been described in the stool of such patients.

Therefore based on the findings of Calgranulin A and B levels being increased in CF sputum compared to control subjects I utilised an in-house ELISA to measure calprotectin in sputum.

5.1.3 Sputum Clara Cell Secretory Protein (CCSP)

In chapter 4 CCSP when measured by mass spectrometry was demonstrated to differentiate all lung diseases from control, in particular CF and bronchiectasis. CCSP has been demonstrated to be lower in BALF fluid from subjects with CF and lung inflammation(275). CCSP has been previously described as decreased in nasal

lavage fluid from patients with allergic rhinitis and increased in the BALF of rats following intravenous LPS administration(276). I therefore sought to reproduce the findings of low levels of CCSP with SELDI TOF MS by utilising a commercially available ELISA.

5.1.4 Sputum Interleukin-8 and Myeloperoxidase

In order to effectively study the utility of the biomarkers discovered in chapter 4 we wished to compare these with more established biomarkers of lung inflammation namely Interleukin-8 (IL-8) and myeloperoxidase (MPO). IL8 has been widely measured in studies of CF lung disease. Levels of IL8 in sputum correlate with the severity of lung function as measured by FEV_1, and can be used to differentiate CF patients from control patients(181, 183, 197, 199, 255, 259). Sputum measurement of IL8 has also been demonstrated to change when patients suffering from CF lung disease are treated with antibiotic therapy(102, 195). IL-8 may be produced by a number of cells in the lung including epithelial cells and monocytes whereas the majority of MPO production is from the neutrophil. MPO is found in the primary azurophilic granules of neutrophils and as such may act as a comparitor to the non-granular calprotectin. Previous studies have demonstrated the presence of myeloperoxidase in the sputum of patients with a range of respiratory diseases, and in particular CF(189, 194, 208, 277). Furthermore sputum myeloperoxidase has been demonstrated to correlate with lung function in CF(189).

5.1.5 Sputum Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) has been reported to be a marker of CF airways disease, being increased in the serum of patients(278). The measurement of VEGF allows a comparison of a novel biomarker described in the literature with those discovered in chapter 4. The source of increased levels of VEGF was postulated as being the (relatively) hypoxic lung tissue of CF patients, so I wished to assess whether increased concentrations could be measured in CF sputum compared to control.

5.1.6 Serum Calprotectin

In chapter 4 I concentrated on the measurement of biomarkers in sputum, however in order to increase the possible clinical application of Calprotectin in CF I assessed whether this was measurable in serum. To this end a collaborating group from the University of Lisbon in Portugal provided us with clinical samples from CF patients and control subjects to facilitate its measurement.

5.1.7 Aims of Chapter

In this chapter I aim to measure levels of novel biomarkers in sputum (and serum), namely calprotectin and CCSP, and compare these to previously described sputum biomarkers from the literature. I hypothesised that calprotectin and CCSP would be superior to previously described sputum biomarkers.

5.2 Methods

5.2.1 Patient Groups

For this section of the study I attempted to validate markers of CF lung disease against a control population. Samples were obtained from CF patients and healthy control subjects as in the previous chapter. Due to the sample limitation I was not able to measure each biomarker in every sample. The number of samples used in each experiment is noted in the results section. Patient samples for serum calprotectin measurement were obtained from the university of Lisbon, Portugal and represented samples from patients attending a local CF clinic with an appropriate diagnosis of CF and control samples. No tandem serum samples had been collected in this study to allow serum measurements in the same subjects as the sputum samples and thus serum samples from this seprate cohort of CF patients were utilised.

5.2.2 Sputum ELISA

An in-house ELISA was utilised to measure sputum calprotectin, please refer to chapter 2 for full methods. IL-8, MPO, CCSP and VEGF were measured using commercially available ELISA kits. For methods please refer to chapter 2.

5.2.3 Serum Calprotectin

Serum Calprotectin was measured by in-house ELISA. The same method was utilised as per sputum aside the sample diluent. For all serum experiments 50% fetal calf serum in DPBS was used as diluent for standards and serum.

5.2.4 Data Analysis

Data analysis was performed on Prism4 (GraphPad, San Diego, USA). Data were subjected to Mann Whitney testing and $p < 0.05$ considered significant. Data were also subjected to receiver operator characteristic (ROC) analysis, to assess the sensitivity and specificity of each test at separating CF from control.

5.3 Results

Data are presented as median (interquartile range) unless otherwise stated.

5.3.1 Subject Characteristics

Samples were obtained from 23 CF patients and 20 control subjects. Serum samples were obtained from 26 CF patients and 28 control subjects. The basic demographics are demonstrated in table 5.1. Unfortunately no lung function data was available for the serum cohort.

Disease	n	Male n	Age years	FEV_1 % pred	Sputum Neutrophil%
Controls Sputum	20	6	36.9 (2.5)	105.8 (2.6)	55(4.3)
CF Sputum	23	14	26.3 (2.0)	59.1 (4.4)	92(2.0)
Control Serum	28	10	32 (2.1)	n/a	n/a
CF Serum	26	15	27 (1.3)	n/a	n/a

Table 5.1 Subject Characteristics For ELISA Biomarker Study

Subjects were recruited from a similar cohort as in chapter 4. Data represent mean (SEM).

5.3.2 Sputum Calprotectin

Sputum Calprotectin was higher in the CF group compared to control (1434 μg/ml [409-2626] vs. 16.65 [8.6-28.6]). This was statistically significant ($p<0.001$). ROC analysis reveals an area under the curve of 0.9932 ($p<0.001$), demonstrating sputum calprotectin to have 100% sensitivity and 95% specificity at a cut off of >49μg/ml in separating CF from control subjects. (figure 5.1).

5.3.3 Sputum Clara Cell Secretory Protein (CCSP)

Sputum CCSP was significantly lower in the CF group compared to control subjects (1790 ng/ml [601-3279] vs.27.2 [9.2-112]). This was significant at $p<0.001$. ROC analysis reveals an area under the curve of 0.9561 ($p<0.001$), demonstrating sputum CCSP to have 95% specificity and 94% sensitivity with a cut off of <310ng/ml separating CF from control (figure 5.2).

5.3.4 Sputum Interleukin 8 ELISA

Sputum IL-8 was increased in the CF patients when compared to control subjects (23.56 μg/ml [10.2 - 44.0] vs. 0.4395 [0.31 - 0.55 μg/ml], $p<0.001$). ROC analysis reveals an area under the curve of 0.9929 ($p<0.001$), demonstrating sputum IL-8 to have sensitivity of 100% and specificity of 95% with a cut off of >1.6 μg/ml differentiating CF from control(figure 5.3).

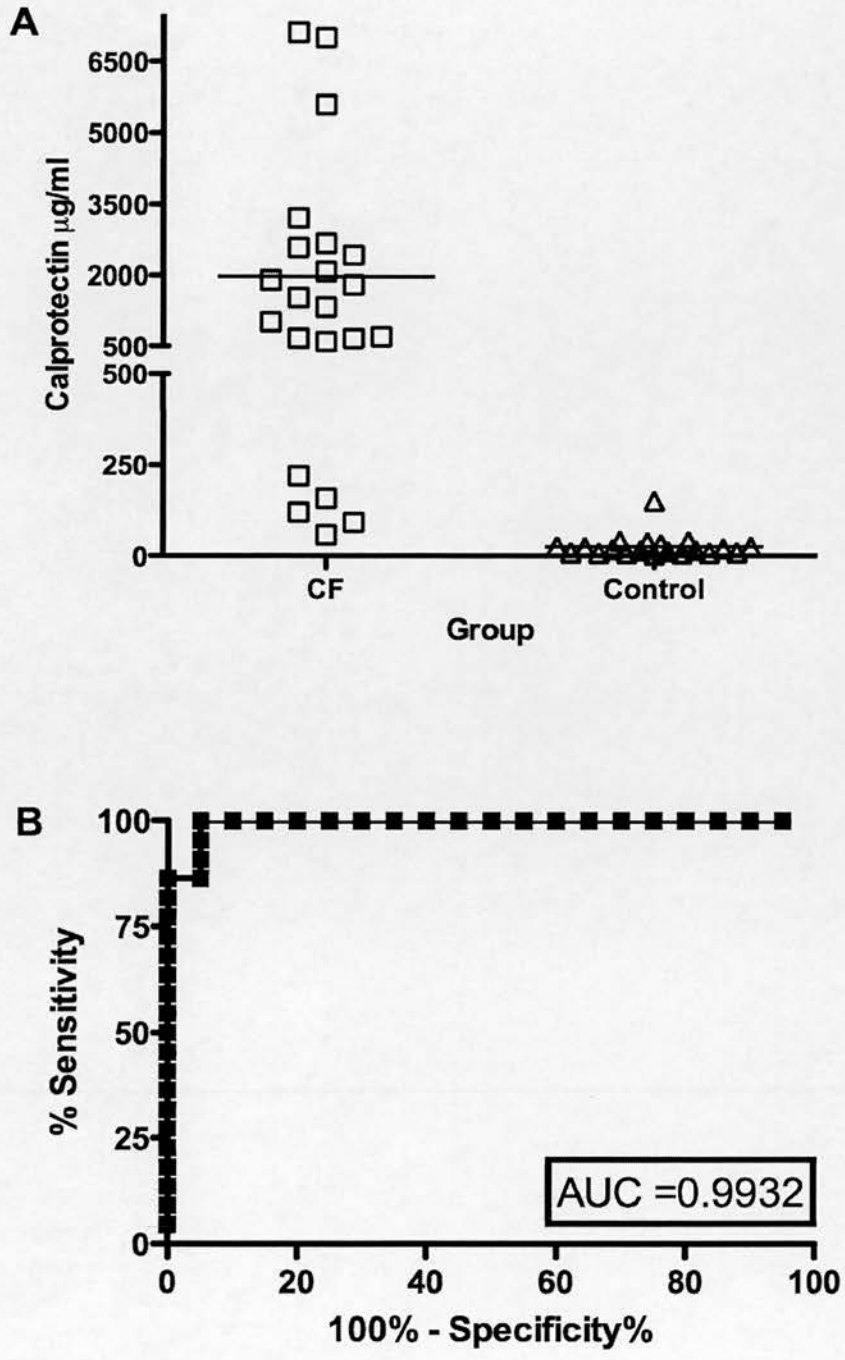


FIGURE 5.1 SPUTUM CALPROTECTIN DIFFERENTIATES CF FROM CONTROL
Sputum samples were subjected to calprotectin ELISA. Panel A Sputum calprotectin is higher in CF patients than in healthy controls ($p<0.001$) [n= 23 vs. 20]. Panel B receiver operator curve analysis demonstrates the high sensitivity and specificity of sputum calprotectin differentiating CF from control ($p<0.001$).

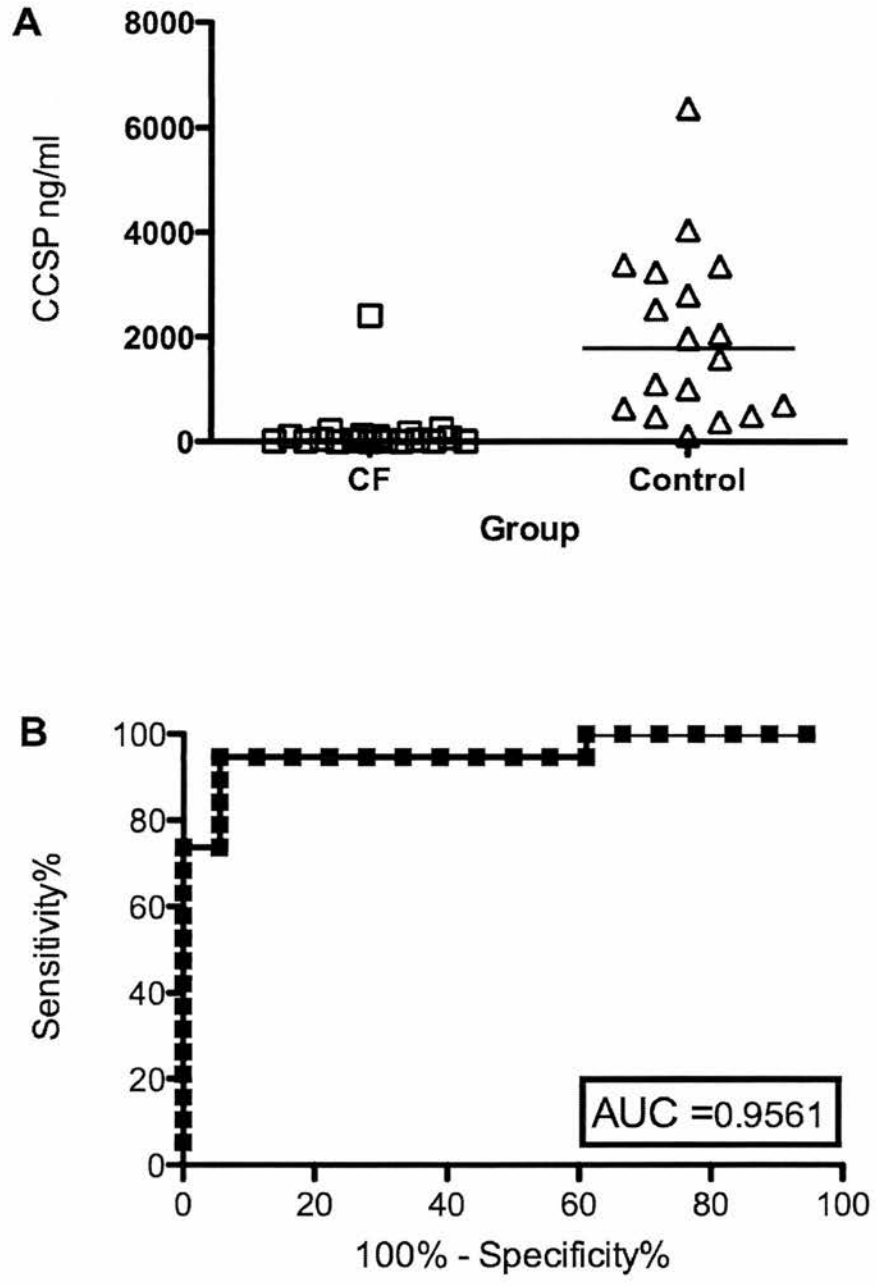


FIGURE 5.2 SPUTUM CLARA CELL SECRETORY PROTEIN (CCSP) DIFFERENTIATES CF FROM CONTROL

Sputum was subjected to ELISA for CCSP. Panel A Sputum CCSP is lower in CF patients than in healthy controls ($p<0.001$) [n= 19 vs. 18]. Panel B receiver operator curve analysis demonstrates the high sensitivity and specificity of sputum CCSP for differentiating CF from control ($p<0.001$).

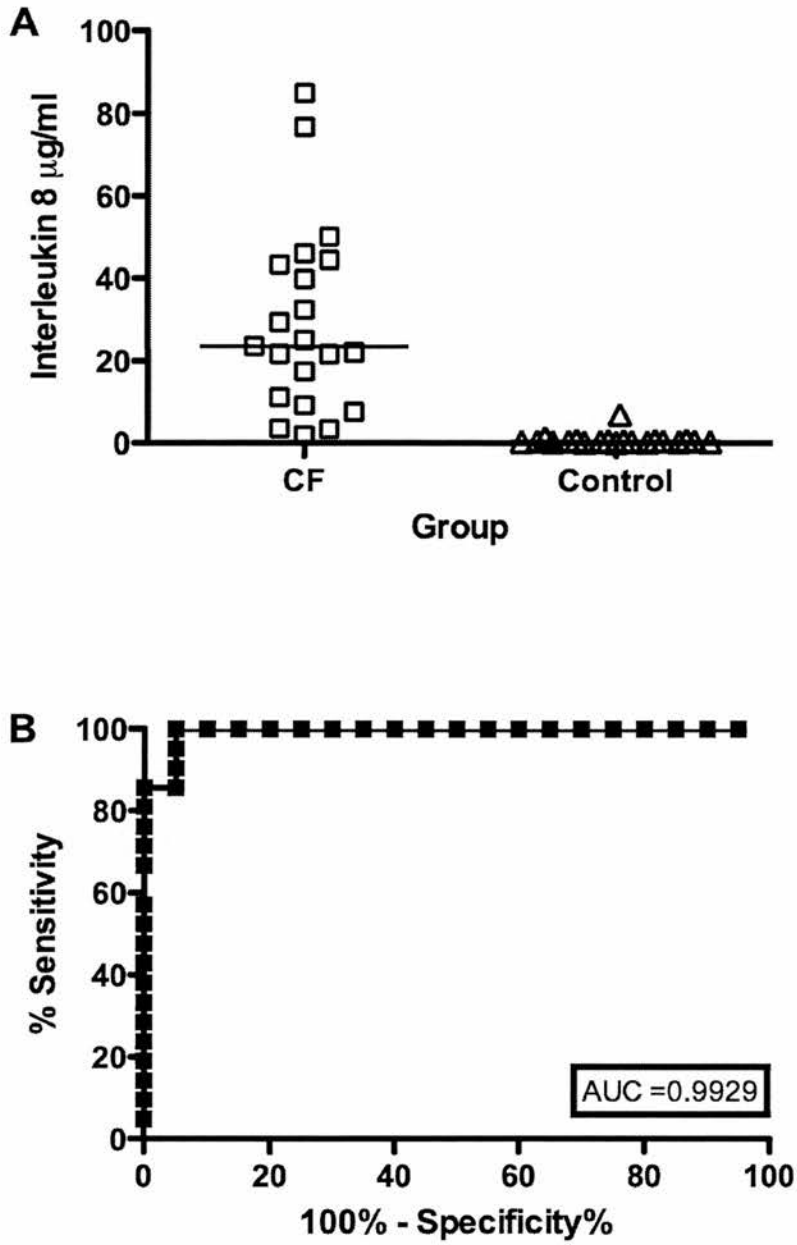


FIGURE 5.3 SPUTUM IL-8 DIFFERENTIATES CF FROM CONTROL

Panel A Sputum IL-8 is higher in CF patients than in healthy controls at $p<0.001$ ($n = 21$ vs. 20). Panel B receiver operator curve analysis demonstrates the high sensitivity and specificity of sputum IL-8 in differentiating CF from control ($p<0.001$).

5.3.5 Sputum MPO ELISA

Sputum MPO was increased in CF patients compared to control subjects (14.44 μg/ml vs. 0.115 μg/ml, $p<0.001$). ROC analysis reveals an area under the curve of 0.995 ($p<0.001$) suggesting 100% specificity and 95 % sensitivity for CF using a cut off value of 0.5 μg/ml.

5.3.6 Sputum Vascular Endothelial Growth Factor

Sputum VEGF was lower in the CF group vs. controls at 133 pg/ml (87.4-171.9) vs. 448.5 (255.6-719.6) at $p<0.001$. ROC analysis reveals an area under the curve of 0.8975 ($p<0.001$). Using a cut off value of <173.6 pg/ml VEGF demonstrates 90% specificity and 80 % sensitivity for CF.

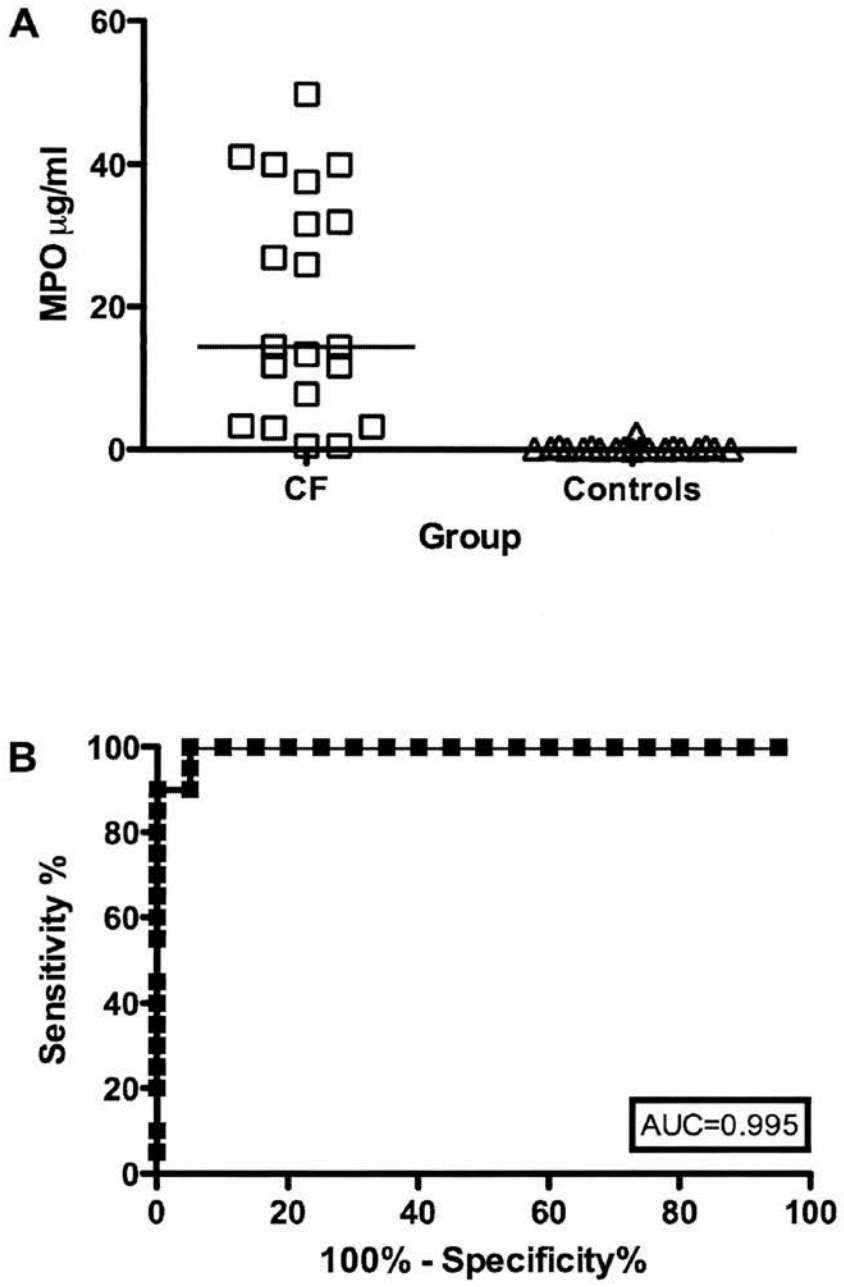


FIGURE 5.4 SPUTUM MYELOPEROXIDASE DIFFERENTIATES CF FROM CONTROL.
Panel A Sputum MPO is higher in CF patients than in healthy controls at $p<0.001$ (n=20 vs.19). Panel B receiver operator curve analysis demonstrates the high sensitivity and specificity of sputum MPO in differentiating CF from control ($p<0.001$).

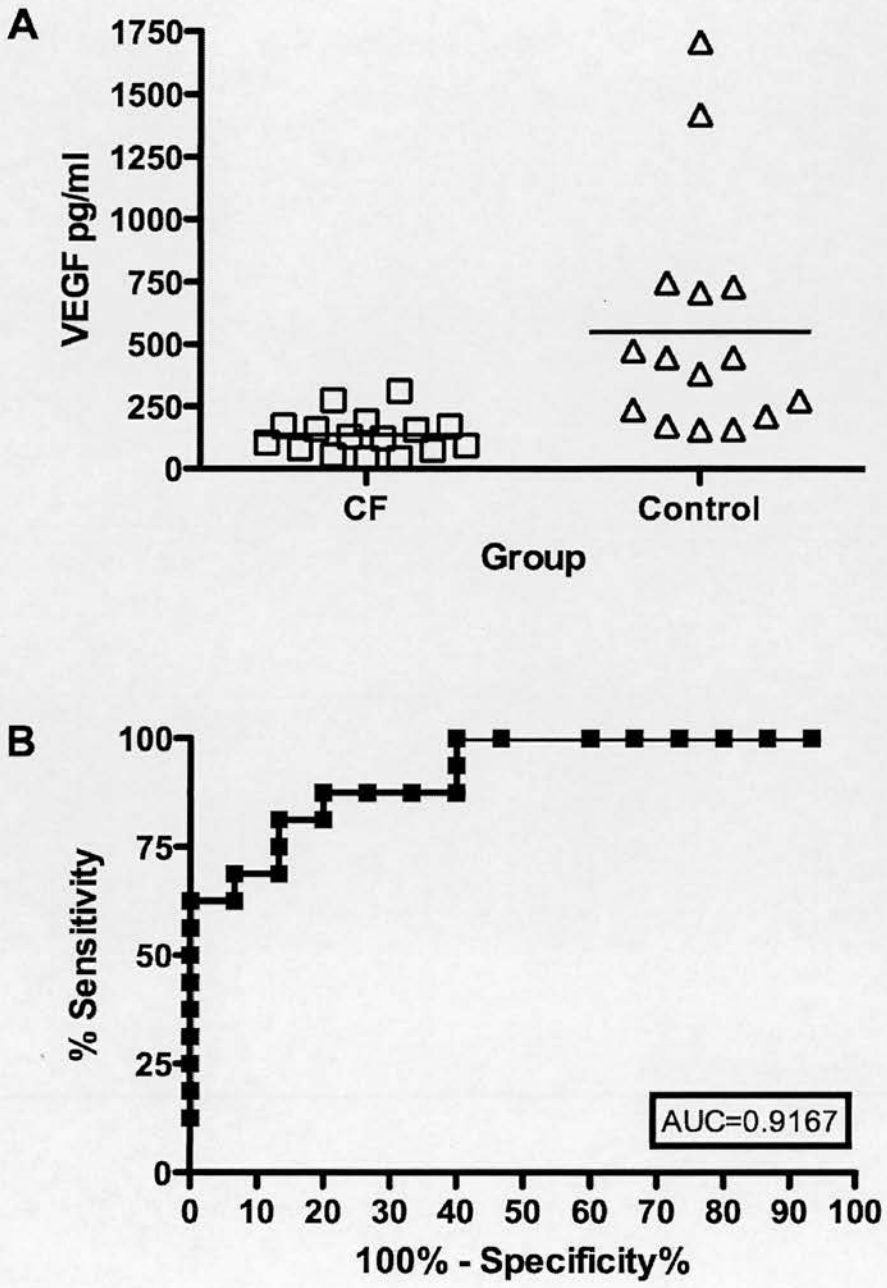


FIGURE 5.5 SPUTUM VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) DIFFERENTIATES CF FROM CONTROL

Panel A Sputum VEGF is higher in CF patients than in healthy controls (n = 16 vs.14).). Panel B receiver operator curve analysis demonstrates the high sensitivity and specificity of sputum VEGF in differentiating CF from control ($p<0.001$).

5.3.7 Correlation of Sputum Markers With Lung Function

No significant correlations were demonstrated between levels of sputum calprotectin, interleukin 8 and myeloperoxidase with FEV_1. There was a significant correlation of between sputum CCSP and lung function. This data is demonstrated in the figure 5.6.

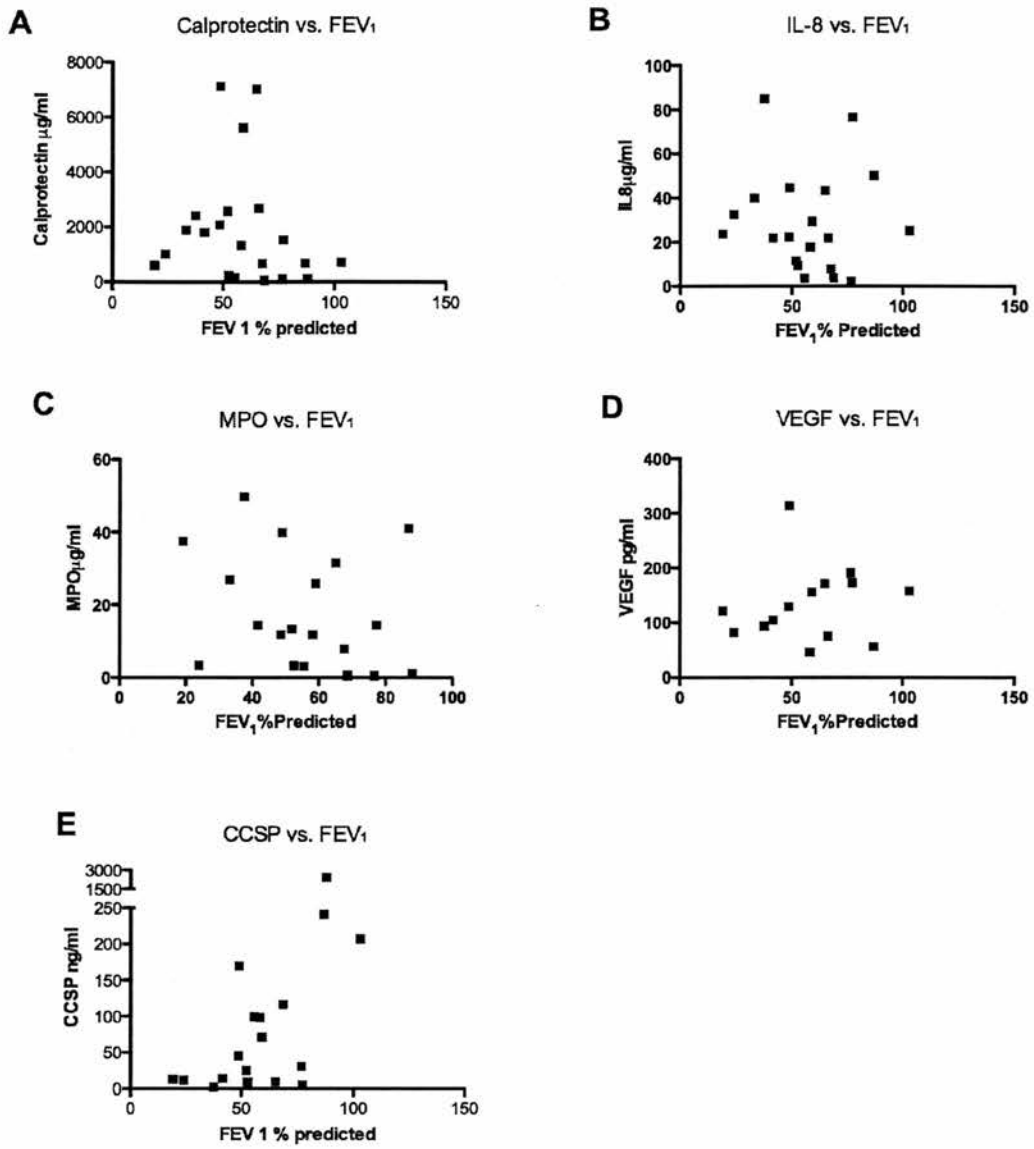


FIGURE 5.6 CORRELATIONS OF SPUTUM BIOMARKERS WITH LUNG FUNCTION IN CF SUBJECTS
A. demonstrates Calprotectin vs. FEV1 (n=22), Spearman r=-0.33, (p=0.145). B. demonstrates IL8 vs. FEV1 (n=21), Spearman r=-0.15, (p=0.537). C. demonstrates myeloperoxidase vs. FEV1 (n=19), Spearman r=-0.35, (p=0.142). D. demonstrates VEGF vs. FEV1(n=14), Spearman r=0.23, (p=0.436). E. demonstrates the only biomarker showing a significant correlation, CSSP (n=18), with Spearman r=0.52 (p=0.025).

5.3.8 Serum Calprotectin

Serum calprotectin was measured in serum samples obtained from Lisbon University, Portugal. The CF and control groups were similar in terms of age. Serum Calprotectin differentiated CF patients from control patients with high statistical significance (9.40 μg/ml [3.5-16.7] vs. 1.72 [1.1-2.9], $p<0.001$). ROC analysis revealed an area under the curve of 0.9025 ($p<0.001$). Using a cut off value of >4.9 μg/ml, serum Calprotectin has 65% sensitivity and 94% specificity in differentiating CF from control.

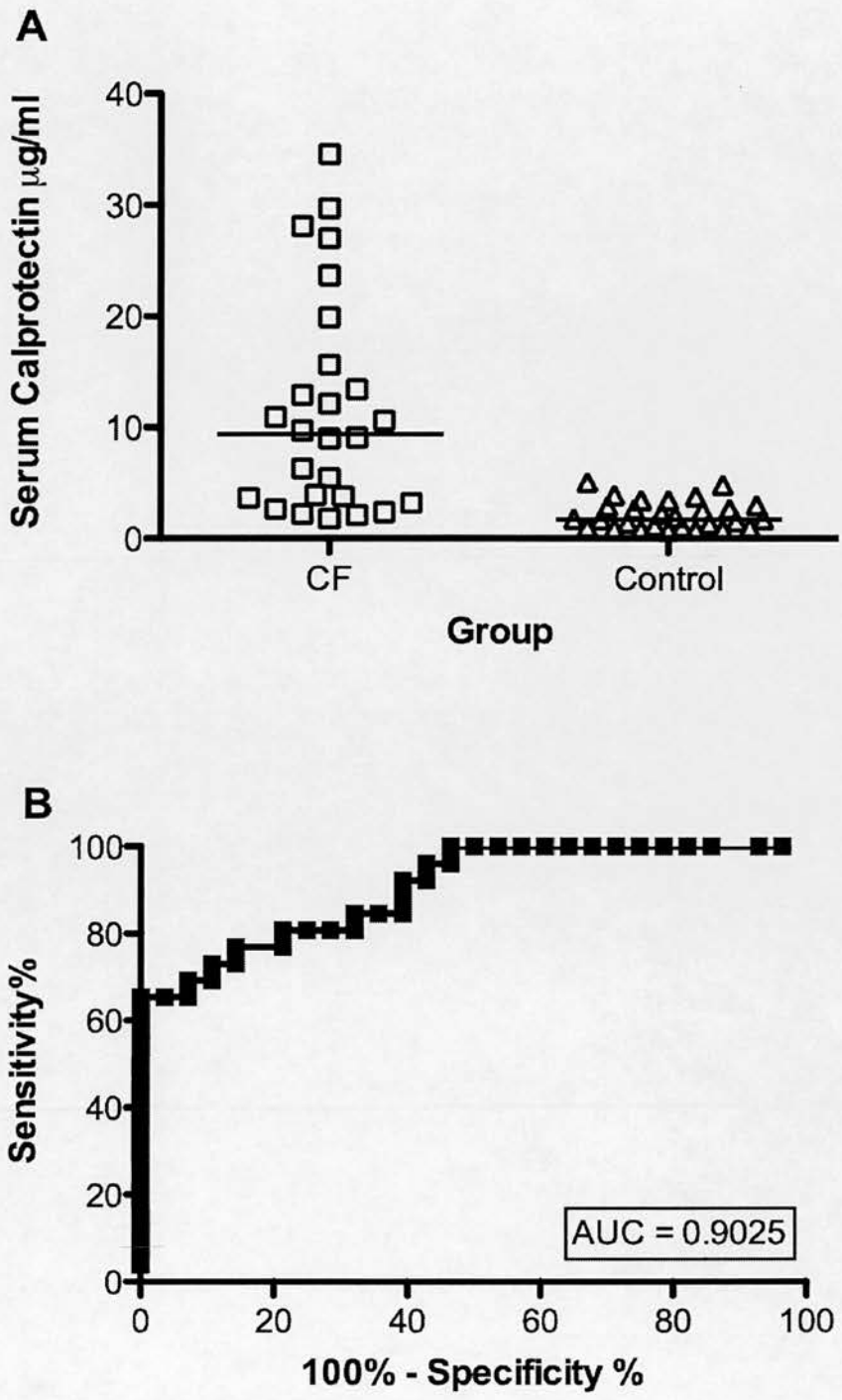


FIGURE 5.7 SERUM CALPROTECTIN DIFFERENTIATES CF FROM CONTROL
Serum was obtained from CF patients and healthy adult controls. Panel A serum calprotectin is higher in CF patients than control subjects (n=26 vs. 28).). Panel B receiver operator curve analysis demonstrates the high sensitivity and specificity of serum calprotectin in differentiating CF from control ($p<0.001$).

5.4 Discussion

5.4.1 General Findings

This chapter demonstrates the application of specific immunoassays to measure protein biomarkers in sputum. As such this chapter represents a natural extension of the findings in previous where several candidate biomarkers were suggested by mass spectrometry. Sputum calprotectin and sputum CCSP were chosen as these were the most differential over- and under- expressed biomarkers in CF sputum. Ultimately the use of immunoassays to assess biomarkers is cheaper and more widely translatable into clinical practice than mass spectrometry, and allows quantification of a given biomarker in a given sample. The use of Reciever Operator Characteristic plots (ROC) allows one to directly compare how well these biomarkers separate a group of control subjects from a group of patients with CF (in terms of area under the curve) and also assess the sensitivity and specificity of this test, allowing the demonstration of calprotectin and CCSP as excellent biomarkers with similar discrimination properties as previously described sputum biomarkers markers. Furthermore sputum calprotectin has a similar discriminating potential for CF lung disease from control to that of faecal calprotectin in distinguishing inflammatory bowel disease from irritable bowel syndrome, a clinical test which is now widely accepted(267).

5.4.2 Sputum Calprotectin

As suggested in the previous chapter calprotectin may be a useful sputum biomarker in CF. The likely source of calprotectin is the airway neutrophil, although it is also produced by macrophages and epithelial cells. Using SELDI TOF MS I described the abundance of calgranulin A and B in CF sputum samples. The heterodimeric

form of these molecules is calprotectin and thus its measurement by ELISA seems relevant. I used an established in-house assay for calprotectin using antibodies and calprotectin standard provided as a gift by Earling Sunderhagen, Norway. These results demonstrate that calprotectin is readily measurable in sputum samples from CF patients and that it differentiates CF from control subjects with a high level of sensitivity and specificity as well as high statistical significance. The results also demonstrate that calprotectin is grossly abundant in the sputum of subjects, in some cases reaching millimolar range. Furthermore calprotectin separates CF from control subjects with a level of sensitivity and specificity similar to previously studied markers of inflammation in CF sputum namely IL8, Myeloperoxidase and VEGF. The potential significance of high levels of calprotectin in sputum is discussed in chapter 4.

5.4.3 Sputum Clara Cell Secretory Protein

CCSP when measured by ELISA can differentiate CF and control patients and furthermore that this correlates with lung function whereas other biomarkers measured in this chapter do not. The correlation of CCSP with lung function in CF patients represents a novel clinical observation and thus merits further investigation. The potential significance of low levels of CCSP in CF sputum was also discussed in chapter 4 and the results in this chapter highlight its potential role as a biomarker in CF.

5.4.4 Sputum Interleukin-8

In this study IL8 is elevated in CF sputum when compared to control values. This has previously been described (193, 197, 199, 255, 259, 279, 280). Previous studies

have demonstrated a relationship between the sputum IL-8 and the severity of lung disease in individual subjects(193, 199). I did not demonstrate any relationship between lung function sputum IL-8. Previous studies were performed however in mainly paediatric populations, whereas the subjects in this study were adults with worse lung function and as such an association with lung function (which is likely to be grossly impaired) may be more difficult to demonstrate.

5.4.5 Sputum Myeloperoxidase

High levels of myeloperoxidase were measured in CF sputum compared to normal controls. These data are in keeping with previous studies in which MPO has been measured (189, 194, 208, 277). This may be predictable as MPO is a major neutrophil protein and thus should be present in large amounts in CF sputum, which contains a high level of neutrophils. As with IL-8 however, there was no correlation with lung function, possibly for the reasons suggested above.

5.4.6 Sputum Vascular Endothelial Growth Factor (VEGF)

VEGF has previously been described as a potential serum biomarker in CF being demonstrated to change informatively during infective exacerbations(278). The source of VEGF was proposed as hypoxic lung tissue in that study, as previous *in vitro* work suggests that VEGF is induced by hypoxia(281). In view of these findings I measured VEGF levels in sputum as a potential biomarker in CF. Contrary to the hypothesis that VEGF levels would be elevated in CF sputum, I demonstrated that VEGF was present in lower levels in CF sputum than control subjects. This finding, although surprising, may simply demonstrate the sampling limitations with

sputum. The level of VEGF measured in the central airway lumen by sputum collection may not reflect changes in the production of VEGF by the pulmonary vascular endothelium. Alternatively the lower level of VEGF in CF sputum may represent either a real decrease VEGF production in CF or a decreased ability to detect this protein in CF sputum. Ultimately paired serum and sputum samples from the same subjects would provide further information, however no serum was collected in this part of the study. Paired measurement in serum and sputum of biomarkers will be commented on in further chapters.

5.4.7 Serum Calprotectin

In this chapter I demonstrate that calprotectin may be measured in serum as in sputum and differentiates CF from control subjects. Serum measurement of calgranulin A has been previously described and led to the description of CF antigen in serum samples from CF subjects(282). Therefore it is reassuring that calprotectin is measureable by ELISA in CF patients, and also in keeping with the previous measurement of calprotectin in the serum of patients with COPD (albeit under the name of L1 protein)(273). As the serum and sputum data are derived from 2 separate patient groups little can be derived from comparisons between the two, and the lack of clinical data from the CF serum sample group would suggest interpretation of this data with some caution. However it does seem evident from the data that the levels of calprotectin measured in the serum are far lower than those measured in sputum. This would suggest that the majority of calprotectin measured in the lung is derived locally from neutrophils and not due to lung leak of serum proteins. Ultimately the measurement of calprotectin in serum may be a more clinically applicable test, as serum samples are relatively easy to collect and need less processing than sputum.

Longitudinal studies are required to assess the application of this assay and will be the focus of chapter 7 in this thesis.

5.5 Conclusions

I have demonstrated the measurement biomarkers discovered in chapter 4, namely calprotectin and CCSP by immunoassay. Furthermore I have shown similar findings to more established biomarkers in CF sputum. I have thus demonstrated in cross sectional data that measurement of calprotectin and CCSP is at least as successful as more established biomarkers in differentiating CF from control with comparable levels of sensitivity and specificity when compared by Receiver Operator Characteristic (ROC) analysis. There were no significant correlations between sputum biomarkers and lung function apart from with CCSP. A potential disparity between the measurement of biomarkers in serum and sputum is suggested in the measurement of VEGF. Further work is required to investigate these findings, such as further cross sectional and longitudinal studies utilising paired serum and sputum samples from the same subjects.

6.0 Sputum Trace Elements as a Biomarker of Cystic Fibrosis Lung Disease

6.1 Introduction

6.1.1 Background

This thesis has concentrated so far on the discovery and validation of protein biomarkers in sputum. As previously noted, sputum cytology has also been employed in previous studies to guide therapy, and the previous chapters have demonstrated the discovery of protein biomarkers in the fluid phase of sputum, but a largely ignored constituent of sputum however is the inorganic content such as metal ions. Trace metals such as zinc are readily measurable in body fluids such as serum but have mainly been used as a markers nutritional status. Furthermore fluctuations in serum levels of trace elements have been described in patients with respiratory diseases, and in particular in CF(283). The presence of trace elements in the lung lining fluid has been less well investigated although increased levels of sputum iron have been described in CF and COPD(229, 230). Conversely a study investigating exhaled breath iron levels in COPD demonstrated lower levels in disease than control(284). Therefore the measurement of trace elements in sputum offers an interesting proposition.

Increased levels of zinc, iron and copper have been reported in abscess fluid(285), and zinc and calprotectin have been demonstrated to co-localise in staphylococcal abscess in a murine model (286), which is of particular relevance to CF considering the high levels of calprotectin measured in the CF airway in the previous chapters. A

major proposed role of calprotectin is as a metal chelater and therefore the interaction of zinc and calprotectin in the CF airway may be of pathophysiological significance.

6.1.2 Trace Metal Ions in Inflammation

Zinc is associated with states of acute and chronic inflammation. Long bone fracture in rats, a classical model of acute inflammation, leads to the accumulation of radio-labelled zinc at the site of induced fractures(287). Furthermore metal ions such as zinc, manganese and copper are obligate co-factors for anti-inflammatory proteins such as superoxide dismutases (SODs) and as such have a close relationship to inflammation(288). Zinc in particular may have a multi-potent role in lung inflammation(289). Metal ions are essential for the growth of pathogenic organisms with the scavenging of free iron being an important component of antimicrobial defence mechanism against organisms such as *pseudomonas aeruginosa*(290). Increased zinc levels in culture media may alter the sensitivity of *pseudomonas* to antibiotics (254), and may also stimulate elastase production in these bacteria (291, 292) further suggesting important roles for zinc in the airways inflammation associated with CF.

Metal ions such as zinc are present in tightly controlled pools in the body and zinc may have multipotent effects in the airway with involvement in apoptotic and anti-oxidant pathways(289, 293), as well as being found in mast cell granules(294). In a mouse model of allergic airways disease, decreased levels of labile (unbound) zinc were recorded in airway epithelial cells(295), and dietary restriction of zinc in these mice led to more significant airways inflammation. Moreover, restriction of zinc in

wild-type rats leads to lung damage with findings characteristic of oxidative stress(296). Interestingly the majority of absorbed dietary zinc is incorporated into metalloenzymes and zinc finger transcription factors(297), thus underscoring zinc's role as an essential micronutrient. Unbound (unincorporated) or labile zinc is available to regulate processes such as cell growth and signalling(298), with labile zinc being demonstrated in airway epithelial cells using specific flurophores(299). Labile zinc co-localises with copper/zinc dismutase, as well as pro-caspase-3 further signifying a role in inflammation and modulation of apoptosis(300, 301). Thus the homeostasis of zinc levels in the airway may be important with elevated levels of zinc and deficiency thereof both having potential effects on inflammation.

The majority of iron in the human body is bound to haemoglobin, myoglobin and cytochromes with more labile stores of iron being bound to iron binding proteins such as transferrin, lactoferrin and ferritin(302). In spite of this tight physiological control, increased levels of total iron and iron binding proteins have been reported in the sputum of patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) in cross-sectional studies, possibly reflecting the level of lung inflammation in these patients(227-230)

6.1.3 Hypothesis

As reported in chapters 4 and 5 of this thesis CF sputum contains higher concentrations than control subjects of calprotectin, an abundant neutrophil protein. Calprotectin is anti-microbial via the chelation of zinc (303, 304) and is found to co-localise with zinc in abscess fluid(286). I thus hypothesised that the sputum zinc

levels would be higher in individuals with cystic fibrosis. As an extension of this hypothesis I supposed that levels of a number of trace metals would also be elevated in CF. Therefore I determined levels of iron, zinc, manganese and copper in the sputum of healthy subjects and CF patients as well as subjects with the inflammatory lung diseases asthma, COPD, and bronchiectasis. Furthermore in the CF group I compared these findings to measurements of sputum protein biomarkers.

6.1.4 Aims of Chapter

The aim of this study was to determine sputum trace metal levels in a number of respiratory diseases and compare the findings in CF subjects with protein biomarkers.

6.2 Methods

6.2.1 Patient Selection

CF and control samples were obtained from the same cohort of individuals utilised in chapter 5 of this thesis, other disease subjects were obtained from the cohort of individuals used in chapter 4. All patients provided informed consent and the study was approved by South East Scotland Research Ethics Service.

6.2.2 Sputum Collection and Processing

Sputum was collected by induction or spontaneously and processed within 2 hours of collection as described in the methods section.

6.2.3 Trace Element Assay

Trace element assay was performed in the Scottish National Trace Element Reference Laboratory, Glasgow, UK. A four-point calibration was used (0, 100, 500, 1000 μg/L Cu, Fe, Zn, Mn in 1% nitric acid). Sputum samples were centrifuged at 800g for 5 minutes and 200 μL of sample was then diluted with 2 ml internal standard solution (100 μ/L yttrium in 1% nitric acid) and mixed by inversion. Internal accuracy was assessed by use of two aqueous certified reference materials TMDA 62 and TMDA 64 (Promochem Ltd, UK). Trace element levels were measured by inductively coupled plasma optical emission spectrometry using a VISTA AX (Varian Limited, UK).

6.2.4 Measurement of Sputum Interleukin 8, Myeloperoxidase and Calprotectin

Sputum measurements for the above biomarkers were obtained from the data set used in chapter 5 and compared to the trace element levels in these patients.

6.2.5 Data Analysis

Data analysis was carried out on Prism4 software (GraphPad, Ca, USA) for Windows. Kruskal-Wallis ANOVA and Dunn's multiple comparison test, $p < 0.05$ was considered statistically significant. Correlations between data were performed using Spearman rank tesing.

6.3 Results

6.3.1 Study Demographics

23 patients with CF, 16 with bronchiectasis, 17 with asthma, 23 with COPD and 20 healthy controls were studied. All samples were collected at a time of clinical stability. Patient characteristics are given in Table 6.1. Of the COPD group 10 were current smokers, 9 ex smokers and 4 gave no information on current smoking status. Of the CF patients 14 were colonised with *Pseudomonas aeruginosa*, the other patients being colonised by a variety of organisms including *Stenotrophomonas maltophilia* and *Burkholderia cepacia complex*.

Disease	n	Male n	Age years	FEV_1 % pred	Neutrophil%
Controls	20	6	36.9 (2.5)	105.8 (2.6)	55(4.3)
Asthma	17	5	51.9 (3.3)	80 (5.6)	60(6.1)
COPD	23	16	66.0 (1.4)	55.0 (4.0)	81(2.6)
Cystic Fibrosis	23	14	26.3 (2.0)	59.1 (4.4)	92(2.0)
Bronchiectasis	16	1	62.3 (2.1)	70.4 (8.5)	84 (4.0)

Table 6.1 Subject Characteristics in Sputum Trace Metals Study

Characteristics for subjects utilised in this chapter. Data are displayed as mean (SEM).

6.3.2 Metal Elements as Contaminants of Experimental Procedure

In order to ensure that trace element levels were not simply a reflection of contamination of samples during preparation, 2 sputum-free sham samples were prepared using identical reagents and procedure (sham samples consisted of 500 μl PBS that was submitted to the same preparation steps as sputum). Zinc, manganese and iron were not detected, but copper was observed as a contaminant at 7.5 μg/L.

6.3.3 Assay Reproducibility

Pooled samples of at least 5 subjects in each group of control, CF and bronchiectasis were assayed in duplicate for zinc levels with CVs of 14.7, 2.7 and 5.4 respectively giving an average coefficient of variation of 7.6%.

6.3.4 Differential Expression of Sputum Zinc and Iron Levels

The absolute concentrations of all trace metals in sputum are displayed in Table 6.2 in μg/L. Sputum zinc concentration was at least 4 fold higher in CF and non-CF bronchiectasis than controls ($p<0.001$). Concentrations in CF and non-CF bronchiectasis were also higher than in asthma and COPD ($p<0.05$) (Figure 6.1). Sputum iron was at least 2 fold higher in CF and non-CF bronchiectasis than controls ($p<0.01$), and COPD ($p<0.05$). Levels were higher in the COPD and asthma groups vs. controls but did not reach statistical significance (Figure 6.2). There was no difference in sputum iron levels between current and ex-smokers with COPD.

Disease	Zinc	Iron	Manganese	Copper
Control	15.35 (10.4-25.6)	13.5 (8.6-21.5)	0 (0-0.25)	8.6 (3-16.4)
Asthma	12.7 (7.2-41.4)	30.0 (6.9-35.3)	0.8 (0.2-1.7)‖	15.2 (8.6-29.5)
COPD	25.4 (9.8-50.7)	21.3 (3.1-35.6)	0 (0-0.7)	15.2 (12.2-22)
CF	135.3 (54.2-209.6)†	56.9 (24.3-115.3) ‖	0.3 (0.1-0.8)	19.5 (14.5-30.1)‡
Bronchiectasis	111.3 (46.1-150.7)†	54.2 (22.7-91.6) ‖	0.6 (0.2-1.3) ‡	15.7 (10.9-33.3)

Table 6.2. Levels of Metal Elements in Sputum Supernatant

Data are displayed as: median (IQR). P values are displayed for disease group vs. control, for p values between groups please consult text.
†$p<0.001$ vs. control
‖$p<0.01$ vs. control
‡$p<0.05$ vs. control

Note: Zinc was below the limit of detection of the assay in 4 control and 1 COPD. Iron was below the limit of detection in 4 control, 1 asthma and 1 COPD. Maganese was below limit of detection in 14 control, 3 asthma, 13 COPD, 6 CF and 1 bronchiectasis.

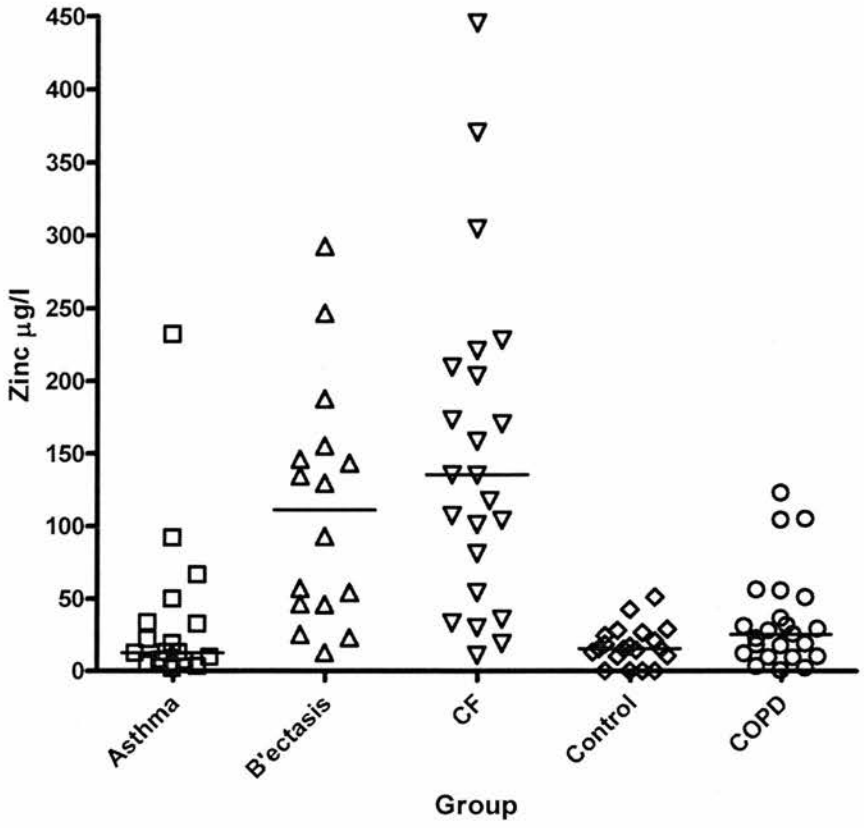


FIGURE 6.1 SPUTUM ZINC LEVELS ARE INCREASED IN CF AND NON-CF BRONCHIECTASIS
Sputum zinc differentiates CF and bronchiectasis from other disease groups and control ($p<0.001$ vs. control, $p<0.05$ vs. other diseases). Sputum zinc is also increased in COPD vs. control but fails to reach statistical significance. Horizontal bars represent medians.

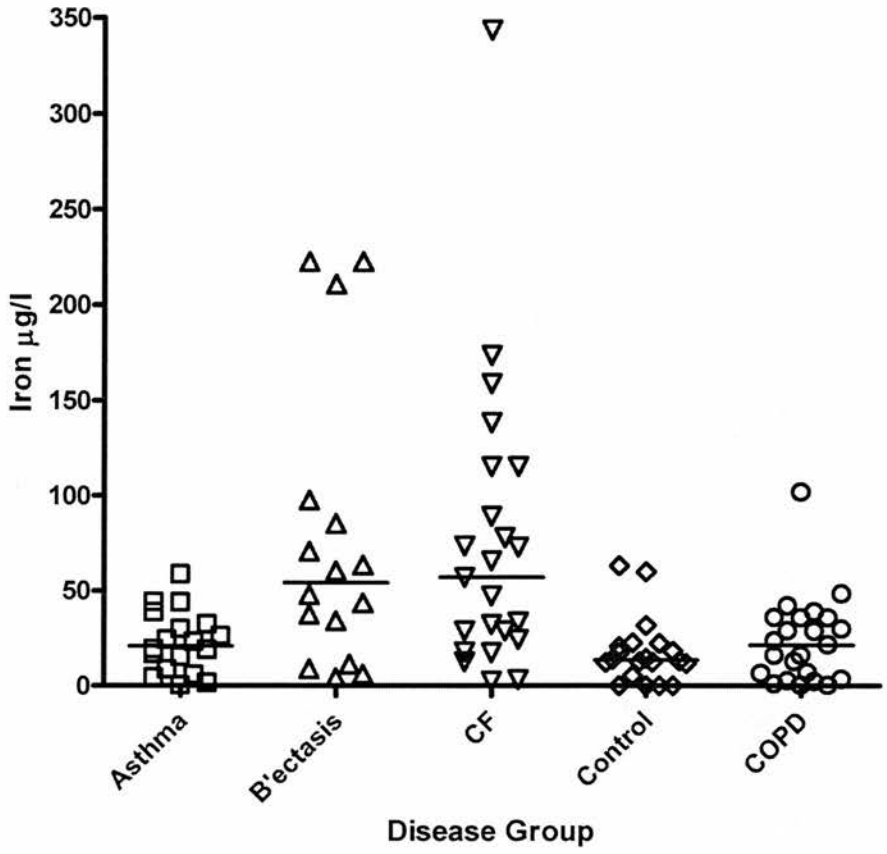


FIGURE 6.2 SPUTUM IRON LEVELS ARE INCREASED IN CF AND NON-CF BRONCHIECTASIS
Sputum iron is raised in CF and bronchiectasis compared to other groups. ($p < 0.001$ vs. control, $p < 0.05$ vs. COPD). Horizontal bars represent medians.

6.3.5 Sputum Manganese and Copper in Cross Sectional Data

Sputum manganese differentiated bronchiectasis but not CF from control ($p<0.05$, figure 6.3). Sputum manganese also differentiated asthma from control subjects ($p<0.01$). Sputum copper was higher in all disease groups vs. control but only reached statistical significance for CF ($p<0.01$) and showed no difference in expression between disease groups. In view of the potential copper contamination of samples this data should be interpreted with caution.

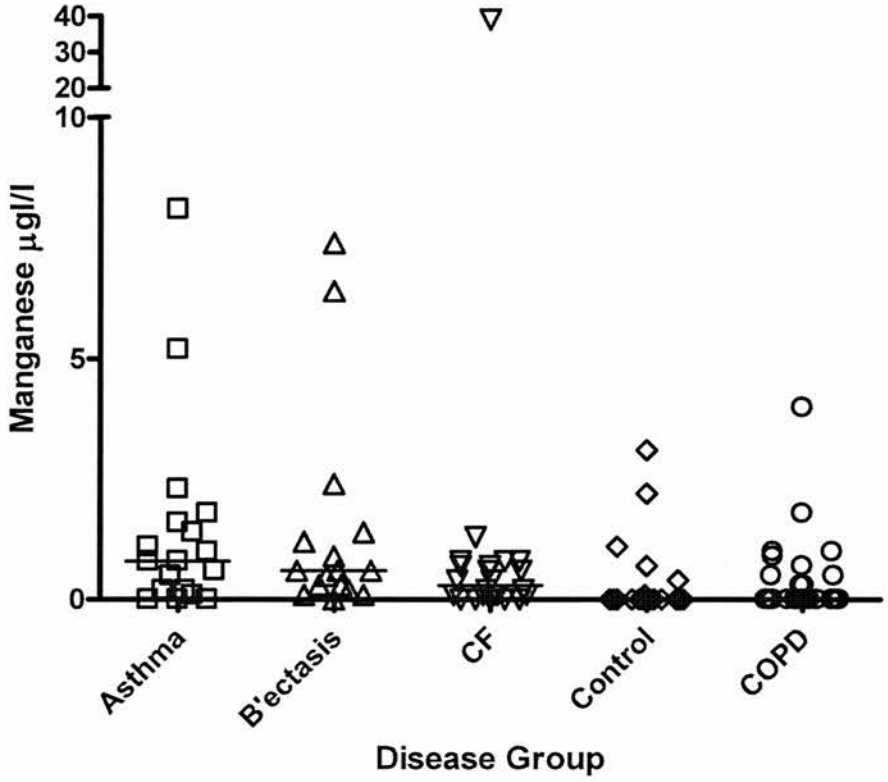


FIGURE 6.3 SPUTUM MANGANESE LEVELS DIFFERENTIATE ASTHMA AND NON-CF BRONCHIECTASIS FROM CONTROLS.

Sputum manganese level differentiated asthma and non-CF bronchiectasis from control subjects ($p<0.01$, $p<0.05$ respectively). Horizontal bars represent medians.

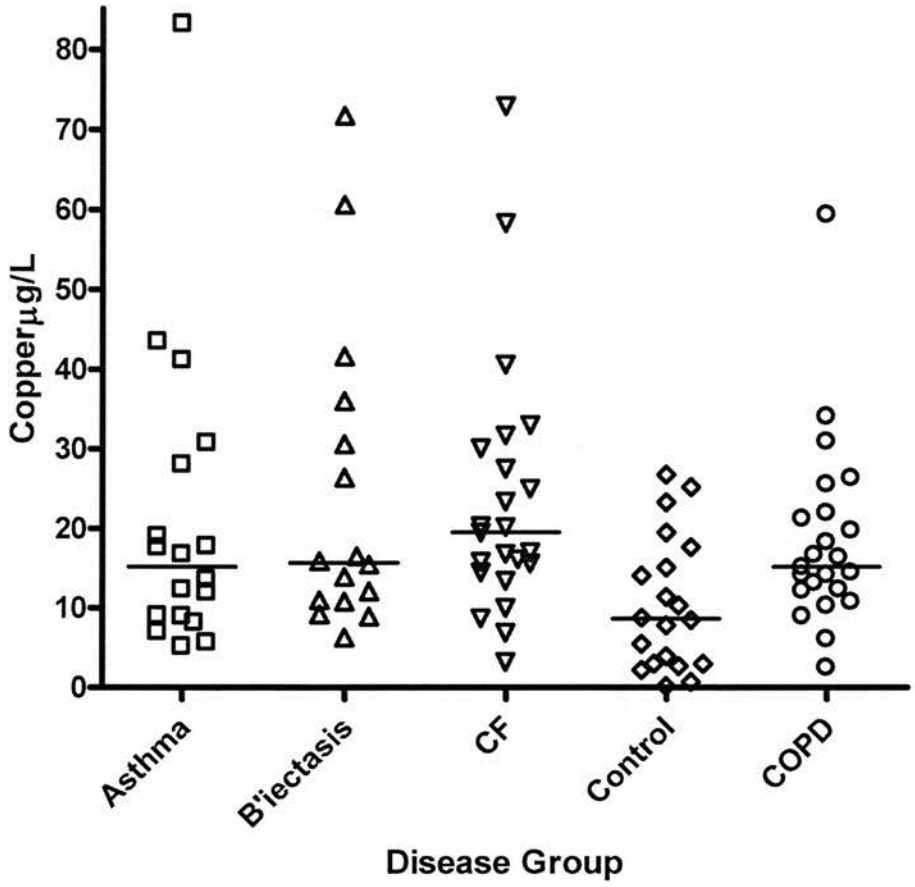


FIGURE 6.4 SPUTUM COPPER LEVELS ARE HIGHER IN ALL DISEASE GROUPS COMPARED TO CONTROL.

Sputum copper level is raised in all disease groups compared to control but only reached statistical significance in CF subjects ($p<0.01$). Bars represent median.

6.3.6 Correlation of Sputum Zinc and Iron Levels with Lung Function

As the most statistically significant changes were seen in the CF group further comparisons were made with clinical data and other inflammatory markers for this group. There was a negative correlation of zinc and FEV_1% predicted in the CF group (*Spearman r*=-0.47, $p<0.05$, Figure 6.5), suggesting that higher concentrations of zinc are associated with poorer lung function. Sputum iron was also negatively correlated with FEV_1% (*Spearman r* = -0.43, $p<0.05$).

6.3.7 Correlation of Sputum Zinc with Protein Biomarkers of Inflammation in CF Subjects

Zinc levels in CF sputum correlated with other measurements of airways inflammation in sputum; myeloperoxidase (Spearman r=0.67, $p<0.001$), interleukin-8 (Spearman r=0.81, $p<0.001$), figure 6.5. Sputum zinc levels correlated most strongly with calprotectin (Spearman r =0.82, $p<0.0001$), figure 6.5.

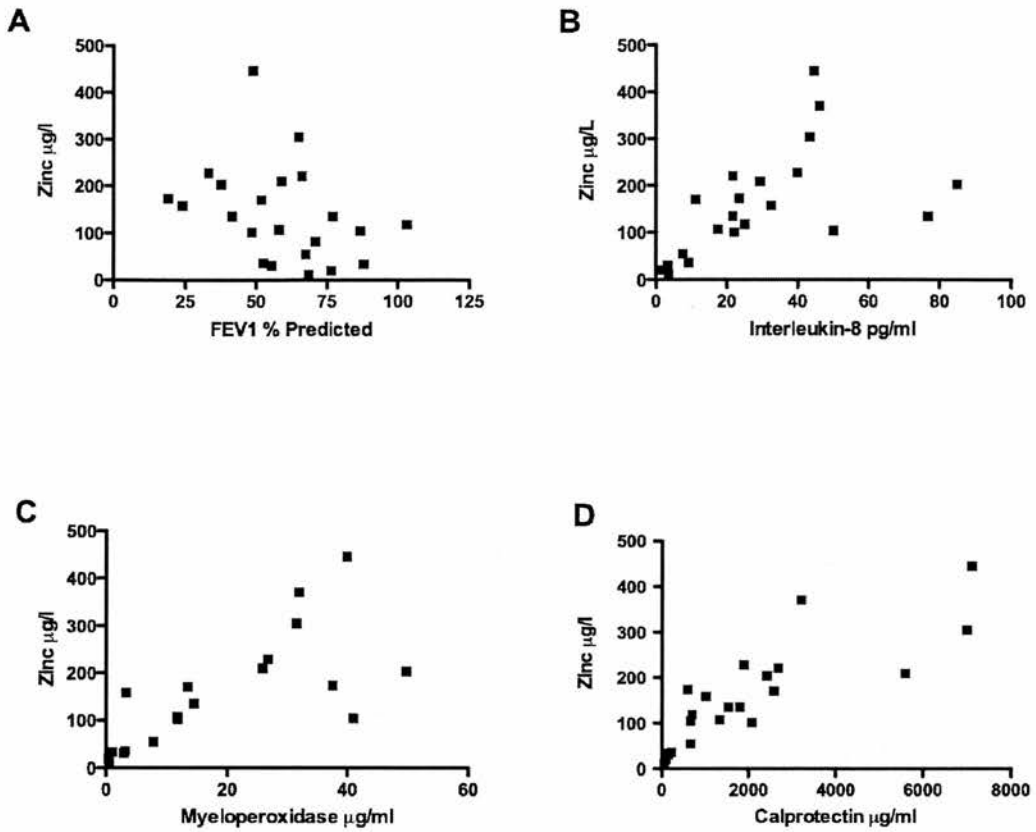


FIGURE 6.5 CORRELATION OF SPUTUM ZINC WITH LUNG FUNCTION AND SPUTUM BIOMARKERS
A. Sputum zinc levels demonstrate a significant negative correlation to FEV_1% predicted suggesting that sputum zinc levels may reflect the severity of underlying lung disease (Spearman r =-0.47, $p<0.05$). B. Correlation between sputum interleukin 8 (IL8) and zinc (Spearman r =0.67, $p<0.001$). C. Correlation between sputum myeloperoxidase (MPO) and zinc (Spearman r = 0.81, $p<0.001$). D. Correlation between sputum calprotectin and zinc (Spearman r =0.82, $p<0.0001$).

6.4 Discussion

6.4.1 General Findings

The results from this chapter demonstrate that levels of elemental zinc and iron in sputum from CF and bronchiectasis subjects are elevated compared to levels seen in healthy controls and subjects with other inflammatory airways diseases. This study also demonstrates correlations between levels of zinc, calprotectin, IL-8 and MPO.

6.4.2 Trace Elements Compared to Protein Biomarkers

I have demonstrated the presence of protein biomarkers and cytokines in previous chapters. However, protease activity in expectorated sputum may affect the robustness of cytokine assays(305). The association between trace element levels and suppurative lung disease demonstrated by the data in this chapter as well the correlation with FEV_1 commends sputum zinc as a robust biomarker. It may also be suggested that trace element measurement would also be less susceptible to serine proteases present in the lung than more established markers such as IL-8 and myeloperoxidase, although this remains speculative and will require further investigation.

6.4.3 Sputum Zinc as a Biomarker and Possible Pro-inflammatory Mediator

Previous studies have investigated the potential use of serum zinc as a marker of lung disease (306, 307), this is however the first study to describe an association between (sputum) zinc levels and inflammation in the sputum of individuals with CF lung disease and the phenotypically similar condition bronchiectasis. Whether elevated zinc has a role in maintaining (or modulating) inflammation or is a by-product of the inflammatory process is less clear. The presence of increased levels of zinc at areas

of inflammation has been described historically(287) and the dietary supplementation of zinc has long been suggested as an adjunct to the treatment of inflammatory skin conditions(308, 309). Zinc may play an important role in modulating the immune response to inflammation, with high concentrations of zinc inducing peripheral blood monocyte apoptosis(310). Zinc may also induce cytokine production such as IL-1, IL-6 and TNF-alpha in monocytes, suggesting a role for zinc in TH1 type inflammatory responses(311). Moreover, zinc has been demonstrated to enhance the stimulatory effects of bacterial lipopolysaccharide on monocyte cytokine expression(312), suggesting an important role in stimulating and modulating inflammation. Conversely low concentrations of zinc may suppress monocyte function and decrease neutrophil phagocytosis(313).

As well as having an effect on circulating immune effector cells, zinc may also interact with the epithelium in airways inflammation(289, 293). Zinc deprivation of bronchial epithelial cells in culture induces apoptosis, which is reversed by zinc supplementation(301, 314), and anti-oxidants(301). In this study I measured the total zinc content of sputum (i.e. bound and unbound) rather than the unbound (labile) content. Labile zinc can be measured in airway epithelial cells and is found in association copper/zinc dismutase and pro-caspase-3 and as such may be important in inflammation and apoptosis regulation(299). Zinc deficiency in a murine model of asthma leads to increased levels of epithelial cell apoptosis and increased levels of airways inflammation on challenge with ovalbumin and is associated with a loss of labile zinc from the epithelium(295). It is unclear how the increased level of zinc in the CF airway lumen demonstrated by the present data relates to the levels of labile

intracellular zinc in the bronchial epithelial cells. This merits further investigation as it may represent an excess of zinc in the airway lumen at the expense of levels elsewhere. These findings could in part explain the low serum zinc in CF subjects compared healthy controls(283). Zinc application to epithelial cells in an animal model can restore chloride transport (the primary defect in CF)(315), and therefore the interaction of the high concentration of airway zinc with epithelial cells may be fundamentally important but ultimately depend on whether the zinc is bound or labile. The measurement of labile zinc in sputum and saliva has recently been described(316) and a comparison of total zinc measured by the method used in this chapter and labile zinc levels may be appropriate.

6.4.4 Sputum Iron as A Biomarker and Pro-Inflammatory Mediator

Increased concentrations of sputum iron have previously been described in CF(227-230). Iron is a pre-requisite for microbial growth, and elevated levels in the CF airway fluid are proposed to be of pathophysiological significance, contributing (for example) to the proliferation of bacteria such as *Pseudomonas aeruginosa* and to systemic iron deficiency through diversion of iron from the circulation(229). Our results are in agreement with a previous study that demonstrated elevated sputum iron levels in CF exceeding those seen in COPD(230). The difference between sputum iron levels in CF and bronchiectasis compared to healthy controls is unlikely to be solely attributable to leak of iron into the lung from the circulation although further work is required to elucidate the mechanism of increased airways iron. Cigarette smoke has also been suggested as a potential source of airways iron(317), but there was no difference sputum iron between current and ex-smokers with COPD

in this study. Thus the source of increased airways iron in inflammatory disease requires further investigation.

6.4.5 Sputum Manganese and Copper

Associations between the levels of sputum manganese and copper with disease type are less obvious, manganese for example was only significantly elevated in the asthma and bronchiectasis groups and copper only in CF. Both metals (like zinc) are co-factors for SODs, which have leading roles in alleviating oxidative stress in the lung(288). In contrast to zinc and iron, they were not as abundant in sputum in our study. Ultimately it is difficult to reconcile the data from these metals with what is known about SOD regulation in airway disease, and in particular with the observation that copper-zinc and manganese SODs may be down regulated in asthmatic airways suggesting sputum copper and manganese are not merely tracking SOD levels. A measurement of SOD level or activity would help to elucidate these complicated relationships but was not performed in this study and thus would form the basis of further work.

6.4.6 Correlation of Zinc Levels with Calprotectin May Be Biologically Significant

Sputum zinc levels correlated with protein biomarkers of airways inflammation namely calprotectin, MPO and IL-8 suggesting its usefulness as a biomarker. Calprotectin is a highly abundant neutrophil protein proposed to have anti- and pro-inflammatory functions, and the ability to chelate zinc and other cations(264, 286, 304, 318). Furthermore calprotectin has been demonstrated promote apoptosis in cell lines via the exclusion of zinc(319, 320). The observation of highly correlated sputum zinc and calprotectin levels may therefore be of importance in the

pathophysiology of CF lung disease. Zinc and calprotectin have been demonstrated to co-localise in *Staphylococcal* abscess in a murine model(286), and calprotectin knock-out mice infected with *S. aureus*, have more marked abscess formation. This work suggested that the majority of calprotectin function was due to the chelation of zinc and to a lesser extent manganese. As such the interaction of zinc and calprotectin in the CF airway may be of mechanistic importance.

Elevated levels of zinc and calprotectin may reflect passive release of the neutrophil contents in view of the large number of nectrotic neutrophils in the CF airway(91), or may represent active sectretion in response to the inflammation in CF and bronchiectatic airways as is observed with lactoferrin release from neutrophils in response to interleukin-1(302). This would certainly be supported by the correlation of zinc with myeloperoxidase, an actively released neutrophil granule protein. Nevertheless, the correlation of zinc and calprotectin might simply reflect an overall abundance of neutrophils, which are rich in both of these substances(321, 322), and may also sugget that the zinc and calprotectin are complexed in the airway. The extent to which the airway neutrophilia characteristic of CF contributes to the excess zinc in the lung is difficult to judge, but it could account for a significant proportion, as neutrophils contain 5-10 ng zinc per 10^6 cells(321). It is also possible that excess sputum zinc may arise due to leakage of zinc-albumin complexes from plasma into the airway as a result of structural lung damage, however this would be difficult to quantify without measuring a marker of plasma exudation, a potential focus in further studies.

6.5 Conclusions

Sputum levels of the trace elements zinc and iron differentiate CF and bronchiectasis from control. Furthermore the trace element copper differentiates CF from controls and manganese levels differentiate asthma and bronchiectasis from the control population in this study. Thus high levels of sputum zinc and iron may be used to differentiate suppurative diseases i.e. CF and bronchiectasis from control populations. Sputum zinc levels are highly correlated with other sputum measurements of inflammation such as IL8 and MPO. The most striking correlation was that of zinc and calprotectin and this may ultimately be of biological significance.

7.0 Longitudinal Monitoring of CF Using Sputum and Serum Biomarkers

7.1 Introduction

7.1.1 Background

CF lung disease is characterised by chronic bacterial infection, beginning in childhood and persisting throughout life. As has been previously discussed, CFTR dysfunction dehydrates the airways lining fluid and decreases mucociliary clearance allowing colonisation with bacteria that rapidly evolve to evade the host defence system(1). CF patients also experience recurrent episodes of increasing pulmonary symptoms termed exacerbations which are often accompanied by a decrease in lung function(98). This may be due to new pathogenic organisms(99) or an increasing burden of already colonising organisms(100). Treatment of chronic of bacterial colonisation with nebulised anti-*pseudomonal* treatment(104) decreases the need for exacerbation treatment as well as improving lung function. Treatment with antibiotic therapy can decrease the bacterial load in exacerbation, as well as decreasing levels of pro-inflammatory mediators(102, 103). As well as bacterial infection other pathogens such as respiratory viruses may be implicated in the initiation of exacerbation, particularly in children(101). The pathophysiology of pulmonary exacerbation in CF is yet to be fully elucidated but it likely combines the host inflammatory response to chronic and acute infection in the already damaged lung, with a number of as yet unidentified triggers.

A major problem in studying the aetiology and pathophysiology of CF exacerbations is the lack of objective diagnostic criteria; indeed no consensus criteria exist in spite

of a definite clinical need(323). Exacerbation has been defined in major CF therapeutic trials from empirical data(104, 105) but these lacked specific objective outcome measurements. In routine practice, clinical judgement and changes in lung function are most commonly used to dictate the need for therapy. In a retrospective study investigating treatment of CF exacerbation, patients were more likely to be treated if symptoms of exacerbation were associated with a loss in lung function and the presence of new pulmonary symptoms and signs(279).

Nevertheless, CF exacerbation provides a model of changing inflammation in CF lung disease. Sputum obtained from CF subjects contains a mixture of proteins which may serve as objective measures of lung inflammation such as interleukin 8 (IL8) (197, 199, 255, 259, 279, 280), myeloperoxidase (MPO) (189, 194, 208, 277), matrix metaloproteinase 9 (MMP 9) (198) and neutrophil elastase (NE) (91, 198, 258). Furthermore NE and IL8 can be inversely correlated to lung function suggesting a relationship of sputum markers to disease severity(193). I have demonstrated similar findings in the previous chapters but have also shown the presence of other biomarkers in CF such as sputum and serum calprotectin and sputum zinc.

Sputum protein profiles(208) and cytokine levels have been demonstrated to change following treatment of CF exacerbations with antibiotic therapy(102, 103). In those studies it is suggested that altering the level of bacterial burden in the lung with antibiotic therapy alters the host inflammatory response. This suggests that exacerbation may be used as an *in vivo* model to study the clinical significance of

new markers of CF lung disease. Other groups have also used exacerbation in CF to demonstrate the presence of novel biomarkers, for example prostaglandin E_2 and cys-leukotrienes, mediators of oxidative stress, were elevated in CF exacerbation compared to stable CF(256). Furthermore serum vascular endothelial growth factor has been reported as a marker of inflammatory change following treatment of CF exacerbation with antibiotic therapy(278). Therefore as well as allowing the assessment of known biomarkers, exacerbation may also be utilised for biomarker discovery experiments similar to those in chapter 4.

7.1.2 Aims of Chapter

The primary aim of this study was to demonstrate whether biomarkers described in previous chapters changed following antibiotic therapy for a CF exacerbation. I therefore investigated changes in sputum and serum calprotectin as well as sputum zinc, iron and copper (I was unable to assess the role of CCSP in exacerbation monitoring due to problems in obtaining the commercial assay). A secondary aim was to compare changes in these biomarkers with changes in lung function and biomarkers suggested previously demonstrated to change with therapy. Additionally I wished to assess the utility of mass spectrometry to discover new markers of CF exacerbation in sputum. This chapter employs CF exacerbation as an *in vivo* model of changing lung inflammation in CF to assess non-invasive biomarkers in sputum and serum samples.

7.2 Materials and Methods

7.2.1 Subjects

Ethical approval for this study was granted by the South East Scotland Research Ethics Service. All patients gave formal written consent. Patients were recruited at the time of a pulmonary exacerbation requiring antibiotics as determined by the clinical team in charge of the individual patient based on the patient having increased respiratory symptoms and an associated decrease in lung function from baseline (for further description please see methods chapter).

7.2.2 Sample Collection

Spontaneous sputum was collected for the assessment of biomarkers at the beginning and end of an exacerbation treatment period, usually within the first 24 hrs of commencing treatment and within 72hrs of treatment cessation. Venous blood was collected by venepuncture and serum separated as in methods section.

7.2.3 Sputum Processing

Spontaneously expectorated sputum was collected from patients and processed within 2 hours of collection as described in previous chapters. Supernatant was stored at -80°C until further analysis.

7.2.4 Measurement of Specific Protein Biomarkers by ELISA

Calprotectin in sputum was assayed by an in-house double antibody sandwich ELISA, using monoclonal and polyclonal antibodies against human Calprotectin (antibodies and calprotectin standard were gifts of Erling Sundrehagen, Norway).

Interleukin-8, myeloperoxidase and VEGF were measured using commercially available kits as described in previous chapters.

7.2.5 Trace Element Assay

Trace elements (zinc, iron, copper and manganese) were measured by inductively coupled plasma optical emission spectrometry (Vesta AX, Varian, Yarnton, Oxford, UK) with yttrium as internal standard as described in previous chapters.

7.2.6 SELDI TOF Mass Spectrometry

A weak cation exchange at pH4 (CM 10) was utilised in view of this surface yielding multiple significant biomarkers in cross sectional studies. Sputum samples were adjusted to contain 1mg/ml protein at a concentration of 0.05% DTT. 10 μL of sample were added to CM10 in a bioprocessor unit (Ciphergen, Freemont, USA). Chips were treated with sinapinnic acid matrix (2x0.8 μL/spot) and allowed to air dry. Samples were analysed on the Protein Biology System 2 SELDI-TOF mass spectrometer (Ciphergen, Freemont, USA). Chips were read with a laser intensity of 210 with deflector set at 4000 Da and a focus mass of 7500 Da following optimisation.

7.2.7 Data Analysis

Paired data analysis of specific biomarkers was carried out using GraphPad Prism software (GraphPad, Ca, USA). For normally distributed data a paired t test was performed. If data was not normally distributed a Wilcoxon sign rank test was used. Data analysis of mass spectral data was performed using Ciphergen Express platform

specific software and then exported to GraphPad Prism for analysis as above. Prior to analysis mass spectral data were pre-processed and normalised to total ion current as described in chapter 2. Mass spectral data analysis was subject to a post hoc bonferoni correction to account for multiple comparisons.

7.3 Results

7.3.1 Patient Demographics and Spirometry

27 individual patients completed the study. Their demographic details are given in table 7.1. FEV_1 was normally distributed pre- and post-exacerbation. There was a significant improvement in FEV_1%(SEM) from 41%(3.3) to 49%(3.6) following treatment as demonstrated in figure 7.1

Patient	M/F	Genotype	Age	FEV_1% Pred Start	FEV_1% Pred End	Colonising Organism	Treatment For Exacerbation
1	f	ΔF508/3659ΔC	20	67	66	BC, SM, HI, SA	TO, CFZ
2	f	ΔF508/ΔF508	30	42	49	PA, SA	TO, CFZ
3	f	ΔF508/ΔF508	20	13	13	PA	CO, MER
4	f	ΔF508/ΔF508	18	55	60	PA	TO, CFZ
5	f	ΔF508/G551D	18	23	39	BC	UK
6	m	ΔF508/UK	46	36	38	PA, SM	TO, CFZ
7	f	ΔF508/ΔF508	21	56	59	PA, SA	TO, MER
8	m	ΔF508/ΔF508	18	58	59	MRSA, SA	TO, CFZ
9	f	ΔF508/G551D	31	31	49	PA	CFZ, CIP
10	m	ΔF508/ΔF508	18	33	43	SA, SM	TAZ, MIN
11	f	ΔF508/ΔF508	20	60	73	PA, Asp	TO, MER
12	m	ΔF508/UK	32	75	88	PA	CIP, AZI
13	f	ΔF508/P67L	27	45	49	SA, BMV	TO, CFZ
14	f	ΔF508/G542X	23	50	82	SA, HI	TO, CFZ
15	f	ΔF508/ΔF508	22	66	75	PA, SA	CO, CFZ
16	f	ΔF508/UK	17	38	44	SA	FL, COAMOX
17	m	ΔF508/G542X	22	56	52	PA	TO, MER, AZ
18	m	ΔF508/G551D	41	24	31	SM, PA, SA. Asp	TO, MER
19	m	ΔF508/ΔF508	24	41	49	SA, PA, SM	TO, CFZ
20	m	ΔF508/ΔF508	37	21	27	BC, PA	TO, MER
21	f	ΔF508/ΔF508	22	45	52	PA	TO, MER
22	f	ΔF508/ΔF508	18	15	15	PA, SA	AZ, MER
23	m	ΔF508/G551D	20	28	28	PA	TO, CFZ
24	f	ΔF508/ΔF508	26	61	64	PA, SM, Asp	TO, CFZ
25	m	ΔF508/ΔF508	17	23	28	PA	CO, MER
26	m	ΔF508/ΔF508	17	33	45	PA, SA	TO, CFZ
27	m	ΔF508/3849+10 kb C→T	22	34	49	PA	TO, CFZ

Table 7.1 Patient Demographics

Patient demographics and lung function from study population. Colonising organisms reflect most recent sputum culture prior to exacerbation. PA=*Pseudomonas Aeruginosa*, BC=*Burkholderia Cenocepacia*, SA=*Staph Aureus*, SM=*Stenotrophomonas Maltophillia*, HI=*Haemophilus Influenzae*, Asp=*Aspergillus Fumigatus*, MRSA=*Methicillin Resistant Staph Aureus*, BMV=*Burholderia multivorans*. Treatment for exacerbation was with intravenous antibiotics apart from subject 12 who received oral treatment. AZ=aztreonam, AZI=azithromycin, CIP=ciprofloxacin, CFZ=ceftazidime, CO=colomycin, COAMOX=coamoxiclav, FL=flucloxacillin, MER=meropenem, MIN=minocycline, TAZ=tazobactam/pipericillin, TO=Tobramycin.

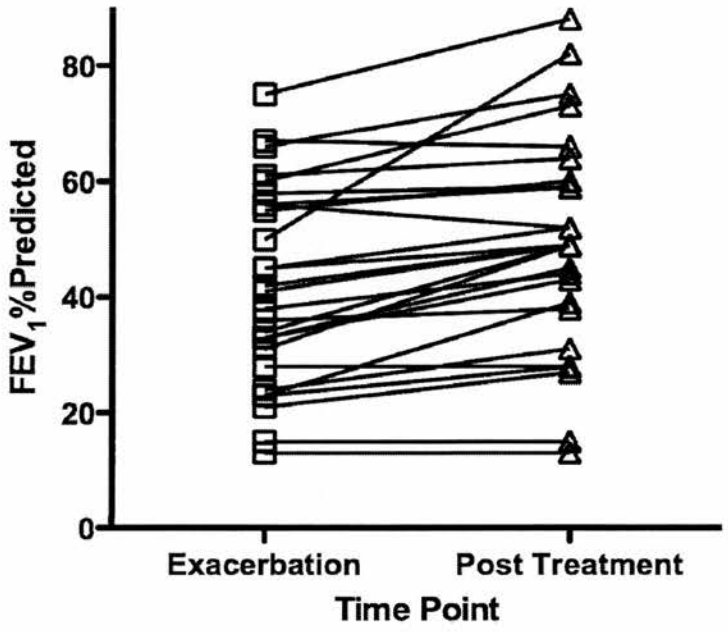


FIGURE 7.1 CHANGE IN SPIROMETRY FOLLOWING TREATMENT OF CF EXACERBATION
Mean FEV_1%(SEM) improved following treatment of CF exacerbation from 41%(3.3) to 49%(3.6) and was statistically significant ($p<0.001$). FEV_1 increased in 22 from 27 patients following treatment.

7.3.2 Changes in Specific Sputum Protein Biomarkers

Due to limitations in sputum sample size not all patients could be assessed for all biomarkers (priority was given to sputum calprotectin which was measured in all 27 paired samples). There was a significant reduction in the level of Calprotectin from median 619.4 (IQ range; 484.1- 971.9) μg/ml to 274.4 (184.0-570.9) μg/ml (p=0.013 figure 7.2). Sputum IL8 and MPO were measured in 26 paired samples (figure 7.2). Sputum IL8 showed a trend to decrease following treatment, from median 30.8 (18.8-53.5) ng/ml to 20.6 (10.3-60.6) ng/ml (p=0.11). Sputum MPO showed a trend to decrease following treatment, from median 41.3 (18.6-49.8) μg/ml to 24.4 (8.8-45.5) μg/ml (p=0.07). Sputum VEGF was measured in 22 paired samples and demonstrated no significant differences before and after treatment 119 (90.5-207) vs. 130.2 (58.5-265.1) ng/ml (p=0.78).

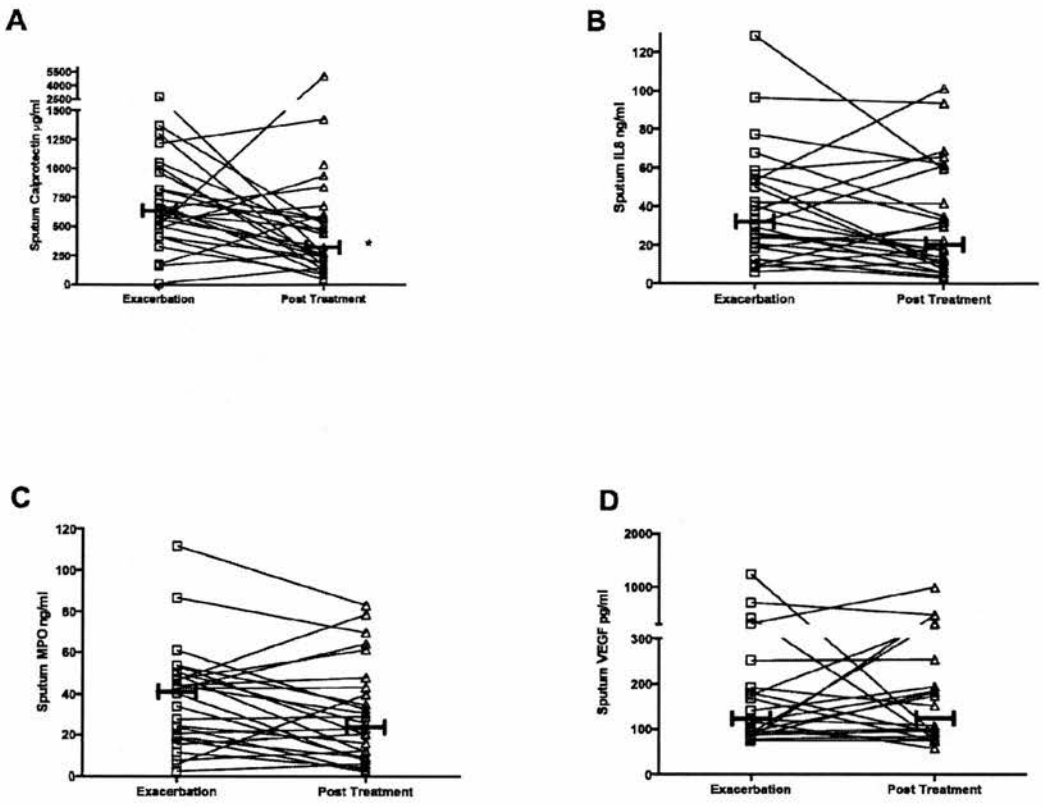


FIGURE 7.2 CHANGES IN SPUTUM BIOMARKERS FOLLOWING EXACERBATION TREATMENT
Sputum biokmarkers were measured by ELISA in spontaneous sputum samples. Samples were collected before and after antibiotic treatment for exacerbation. A. Only sputum calprotectin demonstrated a statistically significant change following treatment of an exacerbation ($p=0.013$). B. Sputum IL-8, C. Sputum MPO, and D. Sputum VEGF did not demonstrate any statistically significant changes following exacerbation treatment. Bars represent median.

7.3.3 Sputum Analysis by SELDI TOF MS

Sputum analysis with SELDI TOF demonstrated significant changes in 6 protein peaks following correction for multiple comparisons. These data relate to 26 paired samples. Four of these peaks represented calgranulin A and B, and 3 are yet unidentified markers at 11061, 9837 and 5295 kDa. The previously identified sputum proteins of calgranulin C and lysosyme C also demonstrated changes between pre- and post- treatment measurements but did not reach statistical significance. These results are displayed in table 7.2 and figure 7.3.

Mass	Protein ID	Median Pre-Antibiotics	IQ Range	Median Post-Antibiotics	IQ Range	p	*p corrected*
5291	UK	3.36	0.8542 -7.352	1.190	0.3017 -2.554	0.0056	*0.2184*
6324	UK	1.72	0.9703 - 3.330	3.262	0.9047 - 6.466	0.0472	*1.8408*
6945	UK	3.594	0.245 - 9.060	7.504	0.6079 - 14.39	0.0137	*0.5343*
7344	UK	5.964	4.132 - 22.12	10.33	4.592 - 21.75	0.0076	*0.2964*
8296	UK	1.292	0.4633 - 2.791	1.922	0.2337 - 6.021	0.0326	*1.2714*
9837	UK	4.589	2.102 - 6.469	0.9538	0.426 - 3.226	0.0001	*0.0039*
10181	Calgranulin C	12.8	8.207 - 18.52	6.54	3.987 - 14.81	0.0071	*0.2769*
10596	Calgranulin A	32.58	24.07 - 46.56	17.73	9.639 - 29.05	0.0004	*0.0156*
10853	Calgranulin A	15.12	9.631 - 33.54	7.187	3.178 - 12.11	0.0001	*0.0039*
11061	UK	2.758	1.494 - 4.599	0.849	0.3649 - 1.794	0.0002	*0.0078*
12720	Calgranulin B	6.684	3.912 - 9.354	2.738	1.541 - 4.331	0.0003	*0.0117*
13196	Calgranulin B	7.374	2.618 - 9.460	2.082	1.045 - 3.238	0.0006	*0.0234*
14679	Lysosyme	15.52	11.36 - 23.43	23.6	14.46 - 44.85	0.0027	*0.1053*
14890	UK	3.001	2.019 - 4.582	5.017	2.886 - 9.279	0.0025	*0.0975*
21468	UK	0.4453	0.2856 - 0.7751	0.2779	0.09721 - 0.5273	0.0027	*0.1053*
26384	UK	0.414	0.2424 - 0.5913	0.2912	0.199 - 0.3635	0.0236	*0.9204*
29313	UK	0.1427	0.07814 - 0.3549	0.3387	0.1441 - 1.192	0.0326	*1.2714*

Table 7.2 Most Discriminating Proteins in Paired Analysis Pre- and Post Treatment of Exacerbation.

Spontaneous sputum samples were subjected to SELDI TOF MS on pH4 CM10 surface. Initial data analysis revealed 39 clusters of proteins for comparison before and after treatment. 17 of 39 protein peaks were statistically significant on paired analysis. 6 protein peaks were considered statistically significant following Bonferoni correction for multiple comparisons including calgranulin A and B (calprotectin). Calgranulins A and B were observed at two separate molecular weights as described in chapter 4.

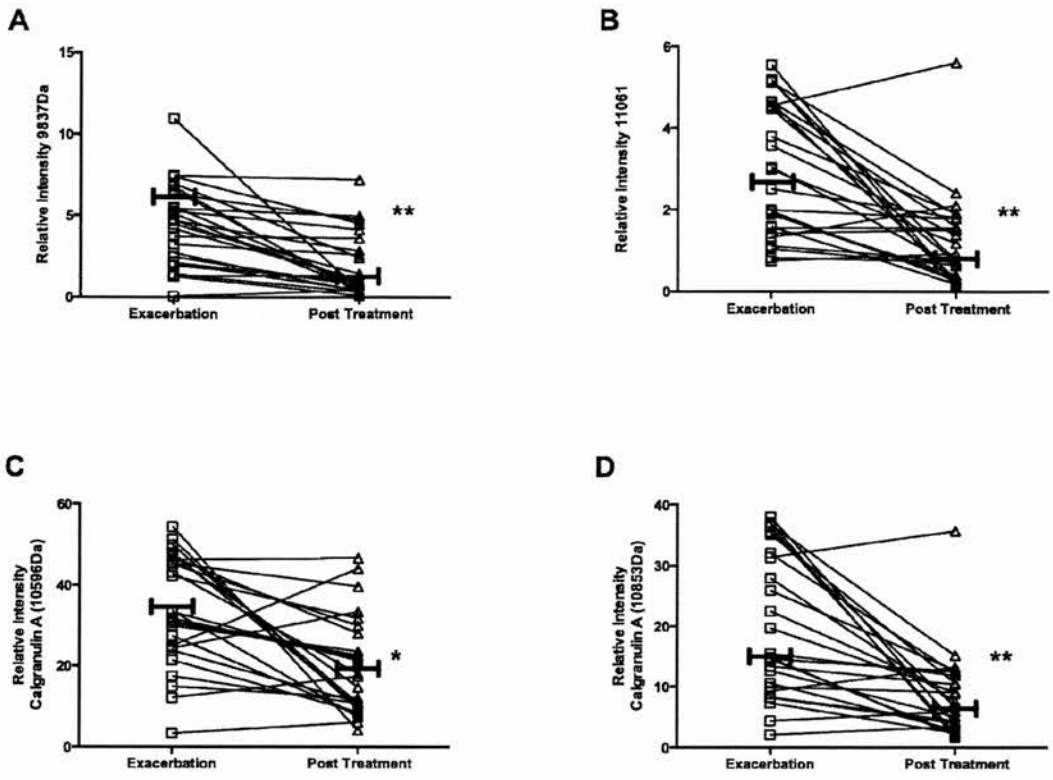


FIGURE 7.3 SELDI TOF MS MARKERS DURING EXACERBATION

Spontaneous sputum was subjected to SELDI-TOF MS analysis. Sputum was collected before and after treament of and exacerbation. A. Unidentified biomarker, molecular weight 9837 Da decreases significantly with treatment ($p<0.01$). B. Unidentified biomarker at 11061 Da decreases significantly with treatment ($p<0.01$). C. Calgranulin A 10596 Da decreases with treatment ($p<0.05$). Calgranulin A 10853 Da decreases with treatment ($p<0.01$). Bars represent median.

7.3.4 Changes in Sputum Trace Metal Levels

Both sputum zinc and iron measurements decreased following treatment of an exacerbation with antibiotic therapy. These results relate to 25 paired samples in view of sample limitations. Sputum zinc decreased significantly following antibiotic therapy from 230.9 (101.4-310) μg/l to 141.4 (53.2-228.6) [p=0.0002]. Sputum iron decreased from 54 (41.05-70.70) μg/l to 49.6 (20.10-76.10), but not significantly [p= 0.1305]. There was no significant change in sputum copper levels from 23.10 (7.3-45.9) μg/l to 20.1 (9.4-54.6) [p= 0.7215]. These results are displayed in figure 7.4.

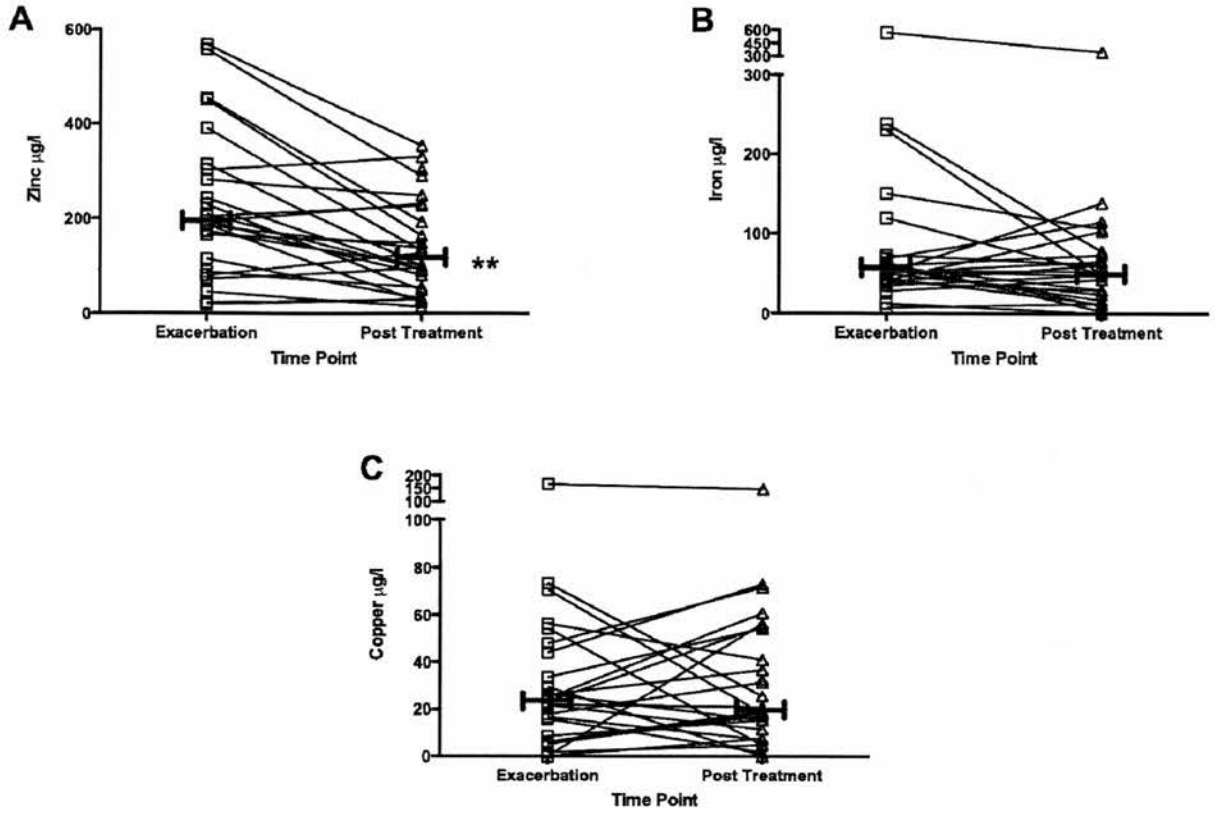


FIGURE 7.4 SPUTUM TRACE ELEMENTS FOLLOWING TREATMENT OF EXACERBATION.
Sputum trace element levels were measured in sputum using optically coupled plasma optical emission spectroscopy in samples collected before and after treatment of a CF exacerbation. A. Sputum zinc decreased with treatment $p<0.001$. Sputum iron (B) and sputum copper (C) did not change significantly. Bars represent median.

7.3.5 Change in Specific Blood Biomarkers

Serum was available in 25 patients from 27 recruited as 2 declined blood sampling. Serum CRP a marker of systemic inflammation decreased from 30.4 (7.6-62.5) mg/l to 6.5 (2.3-20.6) at p=0.002 (measurement relates to 22 paired samples as 3 individual samples were above the limit of detection of the assay: 300mg/l). Peripheral white blood cell count similarly decreased from 11.2 (8.5-16.0) $x10^3$ cells to 8.5(5.2-9.6), this was measured in only 20 patients due to samples not reaching the local haematology lab in time for analysis. CRP and white blood cell count are demonstrated in figure 7.5. Serum calprotectin also decreased significantly over the course of exacerbation from 21.5 (13.3-55.5) μg/ml to 9.3 (6.5-18.2) at p=0.002 (measurement in 25 paired samples), figure 7.6. Serum VEGF, previously suggested as a biomarker of CF lung disease that changes with treatment of an exacerbation, decreased from 385.0 (226.2- 581.5) pg/ml to 236.1 (142.6- 411.9) at p=0.013 (measurement in 25 patients).

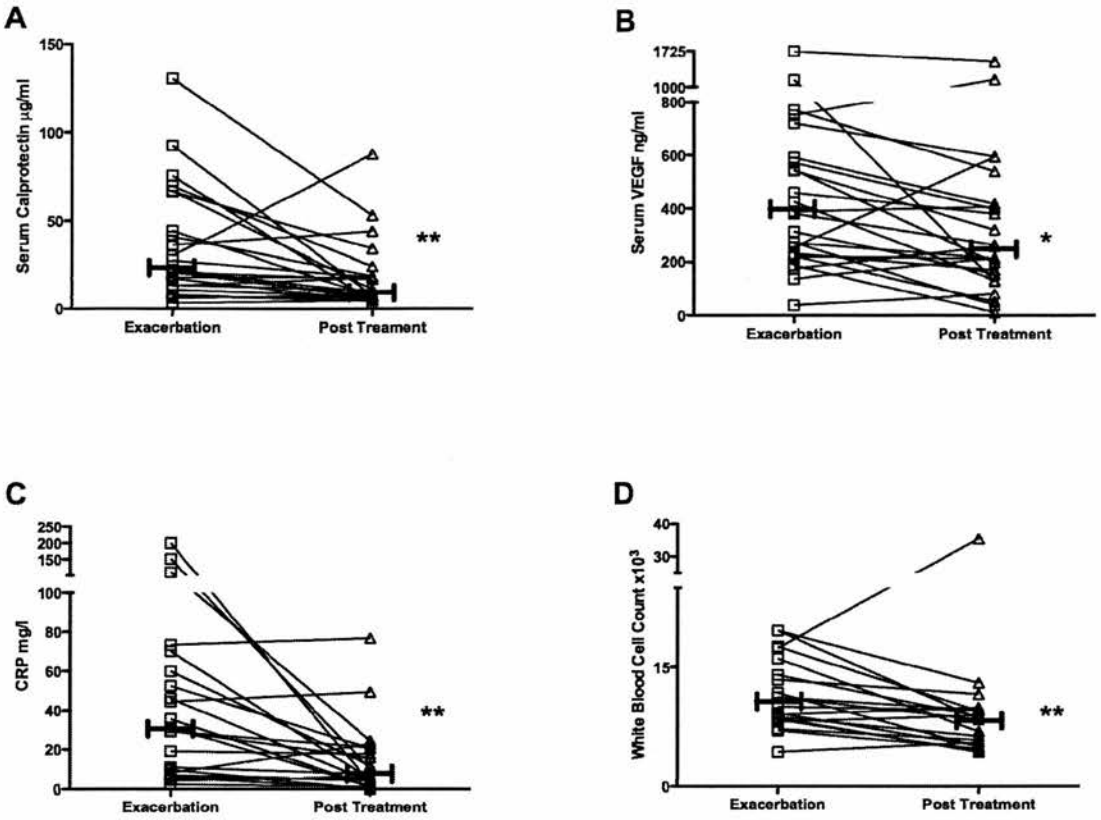


FIGURE 7.5 CHANGES IN SERUM BIOMARKERS FOLLOWING TREATMENT OF EXACERBATION
Blood samples were taken into clot activation tubes and the serum separated. Separate samples were taken for full blood count assessment. Specific ELISAs were used to measure Calprotectin, VEGF and CRP (High sensitivity CRP was utilised with lower limit of detection of 0.1mg/l). A. Serum calprotectin decreases following treatment of an exacerbation at $p<0.01$ (n=25). B. Serum VEGF decreases following treatment of an exacerbation at $p<0.05$. (n=25). C. CRP (n=22) and D. WBC (n=20) decrease following treatment of a CF Exacerbation with statistical significance ($p<0.01$). The high outlier in each group represents a different patient. Bars represent median.

7.3.6 Correlation of Biomarkers With Lung Function

Serum and sputum biomarkers were subjected to correlation with lung function. Table 7.3 demonstartes the correlation of all biomarkers that demonstrated a significant change with exacerbation treatment. Serum calprotectin and sputum zinc demonstrated the most significant negative correlations with FEV_1 (Spearman r=-0.4795, $p<0.001$ and Spearman r= -0.4507, $p<0.001$ respectively).

Biomarker	Spearman r Exacerbation	Spearman r Post-Treatment	Spearman r Overall
Sputum Calprotectin	-0.1933	-0.0775	-0.1718
Sputum Neutrophils	-0.2064	0.1337	-0.09082
Sputum Zinc	-0.5634**	-0.2543	-0.4507***
Sputum 9837 Da Marker	-0.009	0.03569	-0.1218
Sputum 11061 Da Marker	0.2969	0.01373	-0.00714
Serum Calprotectin	-0.4942*	-0.3863	-0.4795***
Serum CRP	-0.3274	-0.2634	-0.3666*
Serum VEGF	0.01393	0.2915	0.08998
White Blood Cell Count	-0.2064	0.1337	-0.09082

Table 7.3. Correlation of Biomarkers Pre- and Post-Exacerbation with Lung Function

Selected biomarkers were correlated with lung function measured by FEV_1 both before and after exacerbation treatment by Spearman rank testing (*$p<0.05$, ***$p<0.001$). Serum Calprotectin ($p<0.001$), sputum zinc ($p<0.001$) and CRP ($p<0.05$), demonstrated a significant correlation with lung function overall. No biomarker correlated with lung function individually both pre- and post-exacerbation treatment.

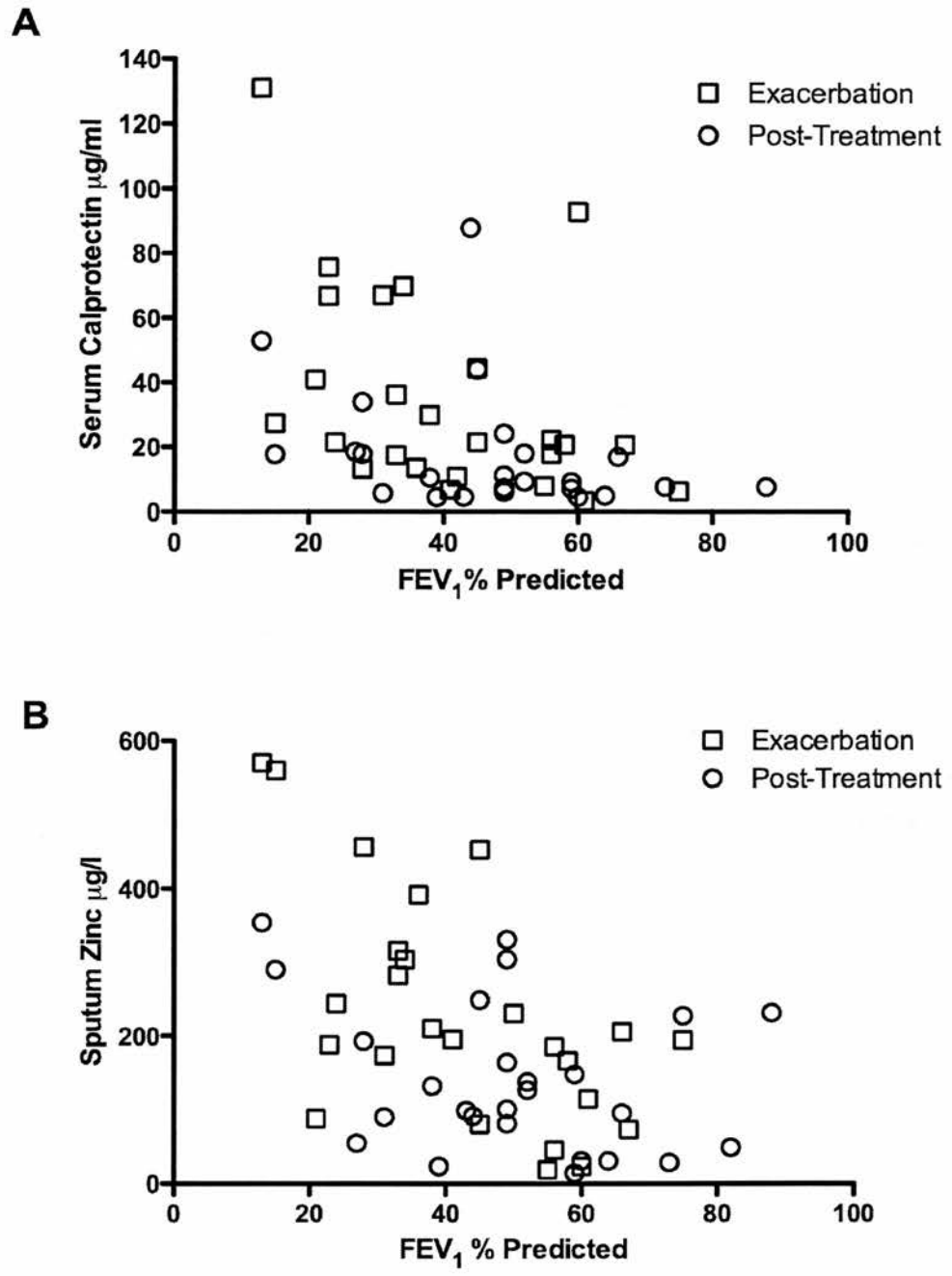


FIGURE 7.6 SIGNIFICANT CORRELATIONS WITH FEV_1

A. Serum calprotectin was negatively correlated with FEV1 at exacerbation but not post-treatment B. Sputum zinc was negatively correlated with FEV1 at exacerbation but not post treatment. For Spearman r values please consult table 7.3.

7.3.7 Relationship of Serum Calprotectin to Time of Next Exacerbation

In order to investigate whether the level of inflammation measured by serum calprotectin at the end of exacerbation would give any useful predictive information patient case notes were reviewed at 1 year following completion of the study and the time to next exacerbation calculated in days from the end of antibiotic therapy. A cut off figure was chosen with reference to serum calprotectin measurements in Chapter 5 where the median value in stable patients was 9.1μg/ml. This divided the group in equal proportions and the median time to exacerbation was significantly shorter in the group with higher serum calprotectin at 70 days compared to 112 days ($p=0.032$). Furthermore 3 patients in the higher serum calprotectin group had died within 18 months of their final study visit. A similar analysis was performed for CRP, using a clinically relevant cut-off of 10 mg/ml but failed to show a difference in the median time to next exacerbation 81 vs. 84 days ($p=0.125$; figure 7.7).

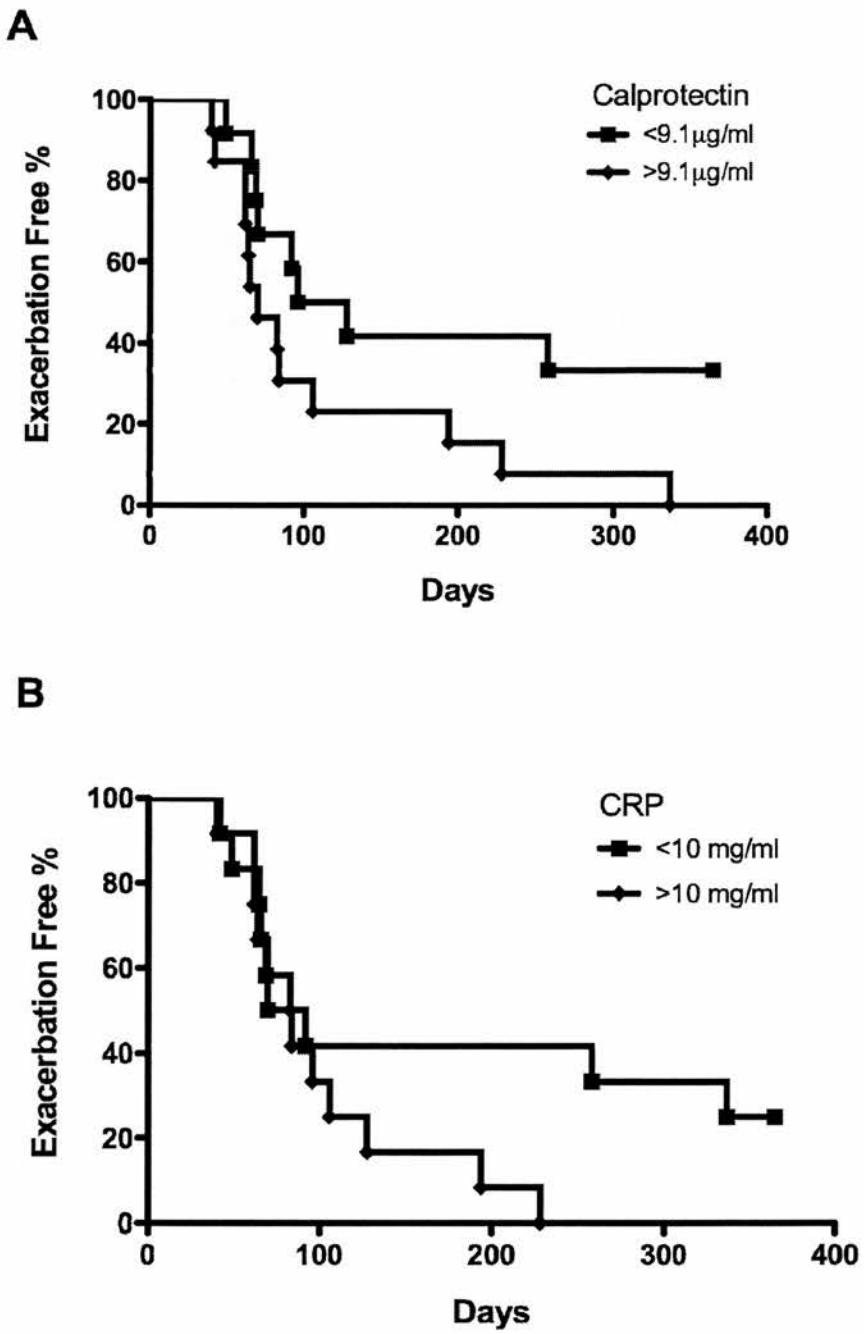


FIGURE 7.7 SERUM CALPROTECTIN BUT NOT CRP PREDICTS TIME TO NEXT EXACERBATION.
Time to next exacerbation from the completion of therapy was calculated for all subjects in the study. A. Subjects with serum calprotectin of <9.1μg/ml at the end of exacerbation treatment have longer time to next exacerbation with median time to exacerbation 128 days vs. 70 days ($p<0.05$). B. Serum CRP of <10 mg/ml did not differentiate time to next exacerbation with median time to exacerbation of 81 vs. 83.5 days ($p=0.125$).

7.4 Discussion

7.4.1 General Findings

In this chapter I have demonstrated that treatment of an exacerbation with antibiotic therapy results in improvements in FEV_1 (as may be expected) and significant changes in several biomarkers in sputum and serum such as calprotectin, whilst failing to demonstrate significant changes in more established markers such as IL-8.

7.4.2 Changes in Pulmonary Function

Several published studies have investigated the effects of antibiotic therapy on pulmonary function, showing that intravenous antibiotics improve FEV_1(99, 102, 103, 324, 325), and suggest that exacerbation may cause reversible changes in the large airways as measured by FEV_1. Furthermore, studies of nebulised antibiotic therapy demonstrate improvements in lung function that result in fewer exacerbations(104). The data presented in this chapter is consistent with the published literature in terms of lung function response, however the patient group utilised in this chapter was older than in previously published articles investigating CF exacerbation (102, 103), which have mainly studied children and younger adults. Therefore the almost uniform improvement in lung function in our patient group may not have been expected, considering that the average % predicted FEV_1 in these patients at the time of exacerbation was 42% predicted compared to 71% predicted in the largest study of exacerbating patients to date(102). The data in this chapter therefore demonstrate that even in a patient population with low baseline lung function FEV_1 is a useful clinical test in assessing the response to therapy and underlines the importance and clinical usefulness of simple procedures such as spirometry in the day-to-day clinical management of patients.

7.4.3 Sputum Calprotecitin

There was a statistically significant decrease in sputum calprotectin following treatment of a CF exacerbation. High levels of calgranulin A and B (the constituent polypeptides of calprotectin) were shown by mass spectrometry and calprotectin by ELISA in CF sputum when compared to control populations in the previous chapters. This chapter firstly demonstrates that calprotectin levels change with antibiotic therapy and secondly that calprotectin in measurable in large amounts in sputum. Calprotectin may be secreted from stimulated neutrophils(326), or released by cell death(322) and as such is an appropriate marker for inflammation in the CF airway, which is predominantly neutrophil mediated. Calprotectin may play an important mechanistic role in the CF airway and has been previously implicated in early lung disease in animal models (75). Furthermore functional knock out of calprotectin in a murine model of pneumonia leads to decreases in inflammatory cell recruitment suggesting an integral role in inflammatory cell recruitment (268). Thus the change in sputum calprotectin following antibiotic therapy implies a direct association of calprotectin with a changing state of airways inflammation. Therefore the data from this chapter demonstrates significant changes in a neutrophil protein in the CF airway following treatment of a CF exacerbation and as such suggests a potential role for this marker as biomarker of response to therapy as well as suggesting a potential role for calprotectin in the pathophysiology of CF lung disease. It should be noted that sputum calprotectin does not change in all subjects and indeed in some subjects it did increase following exacerbation treatment reflecting the complicated biological situation in CF exacerbation.

7.4.4 Sputum IL-8 and MPO

In this study there was no statistically significant change in sputum interleukin-8 (IL-8) and myeloperoxidase (MPO) following antibiotic therapy. Ordonez et al and Colombo et al previously demonstrated a decrease in sputum IL-8 following IV antibiotic treatment(102, 103) and similar findings have been reported following nebulised antibiotic therapy(327). It seems unlikely that this study was underpowered to demonstrate a change in sputum IL-8 (a secondary outcome), as other groups have demonstrated changes in sputum IL-8 with similar sized patient cohorts (103, 327). The largest study demonstrating changes in sputum IL-8 following antibiotic therapy was performed by Ordonez et al, utilising 42 paired samples and demonstrating a modest decrease in sputum IL-8 (0.5 +/- 1.3 log10 pg/ml)(102). Therefore, even if this study was underpowered to detect changes in IL-8 (which seems unlikely), it demonstrates the superiority of sputum calprotectin measurement in this population. One possible explanation for the failure to demonstrate a decrease in IL-8 is that this study utilised an adult population with more severe disease compared to Ordonez et al who excluded patients with an FEV_1 of less than 40%(102). Indeed Downey et al demonstrated no serial change in sputum IL-8 in CF adults following exacerbation treatment, further underlining the possibility that sputum IL-8 is not as powerful a marker in the older patient group(328). This suggests that IL-8 is a less reliable marker in patients with more advanced lung disease and is consistent with the finding that sputum IL-8 is less well correlated to lung function that other sputum markers such as free elastase (193). A further explanation for the differences between this chapter and the study of Ordonez and co-workers was that I utilised spontaneously expectorated sputum and not induced sputum. However, both Colombo(103) and Husson(327) employed

protocols using spontaneously expectorated sputum, and demonstrated similar findings to induced sputum in terms of changes in cytokine profiles.

Although MPO was not investigated in previous studies of CF exacerbation, as a protein released from neutrophils it would be expected to demonstrate a temporal change following treatment of an exacerbation as it has been previously described at high levels in CF sputum compared to control populations(189, 194, 208, 277). The failure to demonstrate a change in this marker may be due to the same reasons that there was no statistical difference with IL-8. The findings of the present chapter are also consistent with a pilot study of oral macrolide antibiotics in CF patients infected with the antibiotic azithromycin which demonstrated no change in sputum MPO following treatment(195), albeit that this study was performed in stable non-exacerbating patients. Furthermore MPO is a primary granule protein in the neutrophil and as such one could postulate its release from neutrophils may be more tightly controlled than that of calprotectin, a cytoplasmic protein.

7.4.5 Changes In Sputum Trace Elements

Sputum Iron has been previously demonstrated to change with antibiotic therapy in a modest number of patients(230). In chapter 6 of this thesis high levels of iron, zinc and copper were observed in CF sputum. Sputum zinc levels changed (decreased) significantly following exacerbation treatment but iron and copper did not. The changes in zinc levels were highly statistically significant, indeed more so than any of the protein biomarkers measured in sputum in this study, and this may reflect the greater stability of zinc in sputum samples and a resistance to protease activity. The

failure to demonstrate a change in sputum iron levels compared to previous work(230) is less easy to explain although could be related to the larger sample size used in this study excluding a previous finding in a small study. As in the previous chapter the similarities in zinc and calprotectin measurement are noted and suggest a possible relationship between these biomarkers and the complicated pathophysiology of CF lung disease.

7.4.6 Changes In Sputum Protein Markers Measured by SELDI TOF MS

SELDI TOF MS demonstrated changes in 6 protein peaks between pre- and post-treatment of an exacerbation. SELDI TOF MS was utilised in earlier chapters to discover biomarkers in CF sputum in cross sectional samples. From this previous work I was able to classify 4 of the significantly different peaks as calgranulin A and B (calprotectin). Interestingly there was no significant representation of a protein peak representative of CCSP, one of the key biomarkers discovered in previous chapters, this may be due to the very low levels of this protein at the time of exacerbation as in the stable CF patient it is already markedly lower than healthy controls. The two remaining peaks were at 9837 and 11601 Da and further identification and characterisation is required to ascertain the significance of these peaks. A greater number of peaks were statistically significant prior to correction for multiple comparisons, including lysosyme c and as such applying the Bonferoni correction to our MS data may have increased type 2 error leading to rejection of actual biomarkers of exacerbation(329). Only 1 ProteinChip surface was selected for the comparisons in this study as the CM10 cation exchange selectively binds biomarkers that have been previously identified in sputum and can be performed

reproducibly. Therefore SELDI TOF MS confirmed the findings of temporal changes in calgranulin A and B (calprotectin) as well as suggesting some future target proteins for identification as biomarkers.

7.4.7 Changes in Serum Calprotectin and VEGF

Serum calprotectin decreased following exacerbation treatment. This finding was of higher statistical significance than calprotectin in sputum suggesting that the measurement of calprotectin in serum is less variable than in serum. Calgranulin A (sub-unit of calprotectin) has previously been described in the serum of homo- and heterozygotes with CF mutations(282) and was previously known as CF antigen. In this study the changing serum levels of calprotectin, suggest a potentially important role for calprotectin in the complicated biology of CF exacerbation. The serum levels of calprotectin are approximately 4 fold less than those observed in sputum suggesting that the high concentrations of calprotectin observed in sputum are likely to arise from local excretion into the airways as an active process from neutrophils, macrophages and epithelial cells or possibly by release from necrotic neutrophils which are more prevelant in the sputum of CF patients with gram negative infection (91). Furthermore the levels of calprotectin in serum could reflect increased neutrophil recruitment and activity from the bone marrow or backwash of calprotectin from the lungs into the systemic circulation due to a breakdown in epithelial barrier intergrety, although this would require further study.

I have also demonstrated that serum calprotectin concentrations of less that 9.1 μg/ml following exacerbation treatment are a good prognostic indicator for patients in terms

of time to next exacerbation, and furthermore that this is superior to measuring CRP in these patients. The cut-off value was representative of the median calprotectin level in the serum of CF patients from chapter 5, and as such represents a clinically relevant endpoint.

Serum VEGF has previously been demonstrated to decrease with treatment of a CF exacerbation with the suggestion being that VEGF is produced by hypoxic lung tissue(278). Increased levels of serum VEGF have also been reported in other lung diseases such as tuberculosis and severe obstructive sleep apnoea(330, 331). The present data demonstrates a decrease in serum VEGF following treatment of an exacerbation but no corresponding change was observed in sputum VEGF, suggesting that the high levels of serum VEGF that change with exacerbation treatment are not derived directly from the central airways. VEGF production has been demonstrated in vitro in hypoxic alveolar cells(281), and therefore the measurement in sputum may be underestimate the level of VEGF being produced in the peripheral airways and alveoli. Nevertheless this study confirms that serum VEGF may be used as a surrogate marker of response to exacerbation. Further investigation would be required to assess any potential mechanistic role in CF lung disease.

7.4.8 Changes in CRP and WCC

This study demonstrates changes in standard clinical measurements of systemic inflammation, namely C reactive protein (CRP) and white blood cell count (WCC). These measurements may be expected to change with therapy of an exacerbation

with antibiotics as decreasing the bacterial load in the lung may decrease the amount of inflammation measurable systemically. These findings are in agreement with those of Colombo et al who demonstrated a decrease in both CRP and WCC following intravenous antibiotic therapy(103). Unfortunately only limited conclusions can be drawn from the data relating to white blood cell counts as these were measured in the local haematology laboratory and only 20 subjects had paired measurement due to some samples being discarded prior to analysis following delays in reaching the laboratory.

7.4.9 Limitations of Present Study

Although presenting a number of interesting clinical observations this study also presents some caveats. Firstly no measure of quantitative microbiology was taken pre- and post- antibiotic therapy as in other studies of exacerbation(102, 103). Therefore the effectiveness of antibiotic therapy at reducing bacterial burden is assumed by the clinical response of patients to therapy. Moreover the usefulness of quantitative bacterial culture as an outcome may be questionable due to difficulties in its interpretation in this more complicated adult population. Furthermore this study was designed to assess the usefulness of biomarkers in monitoring serial changes in subjects rather than to assess the efficacy of antibiotic therapy.

This study was performed on spontaneous samples of sputum. This sampling modality was employed to reduce the sampling burden on participating subjects, as the majority of adults with CF lung disease will spontaneously expectorate sputum. Data from chapter 3 demonstratesthe similarities in sputum profiles between

spontaneous and induced sputum on mass spectrometry and as such suggests equivalence. Furthermore Colombo et al employed a protocol of using spontaneous sampling giving similar findings to those from an induced sputum study of exacerbations(102, 103). Ultimately by employing spontaneously expectorated sputum as a sampling modality in adult CF patients, this allows clinical tests to be performed without the need for more invasive and time consuming procedures that may impact on patient care. It is worth noting however that the sampling of induced sputum may be required in children with CF lung disease or adults with mild disease to perform this sort of study.

7.5 Conclusions

This study demonstrates a number of important clinical observations. A number of sputum and serum markers change significantly during the treatment of infective exacerbations, and therefore could be used to monitor such events. Sputum and serum calprotectin were the most informative protein biomarkers and appeared superior to sputum IL8 and serum CRP, both of which have been favoured hitherto. The level of serum calprotectin following treatment of an exacerbation may also predict the time to next exacerbation. Sputum levels of zinc change informatively following treatment of an exacerbation with high statistical significance. I would suggest that calprotectin and zinc may interact *in vivo* as they do *in vitro*, and as such the role of calprotectin and zinc in CF lung disease merits further investigation. Finally this study demonstrates two, as yet unidentified sputum protein markers, that when measured with mass spectrometry, change significantly following exacerbation treatment.

8.0 Conclusions and Suggested Further Investigations

8.1 Biomarker Discovery and Validation in CF

In this thesis I have described the application of mass spectrometry to the discovery of protein biomarkers in CF lung disease, as well as utilising techniques to discover non-protein biomarkers in CF. This will allow the development of clinically relevant biomarkers, in particular calprotectin, for further validation in CF populations and ultimately into clinically relevant assays.

8.1.1 The Use of SELDI TOF MS in Biomarker Discovery

Chapter 3 on this thesis describes early experiments employed to validate and assess the reproducibility of SELDI TOF MS to investigate biomarkers in CF sputum. SELDI TOF MS is reproducible in both patient and control samples and large amounts of information regarding protein profiles may be gained from using relatively few chip surfaces. Furthermore, spontaneously expectorated sputum and induced sputum result in statistically similar protein profiles on mass spectrometry.

In Chapter 4 SELDI TOF MS is utilised as a high throughput screening tool to differentiate patients with CF lung disease from control subjects and patients with other lung diseases. Protein profiles were similar in CF and non-CF bronchiectasis but were markedly different from controls, asthmatic patients and COPD patients. The most discriminatory biomarker between CF and other groups (bronchiectasis excluded) was calgranulin A (part of the calprotectin heterodimer). Further proteomics and western blotting confirmed this identity. Furthermore a lower

molecular weight version of calgranulin A was characterised from the sputum of CF subjects, a finding that has since been described in a study using CF BALF(175), and may have mechanistic implications.

8.1.2 The Measurement of Calprotectin and Other Biomarkers by Immunoassay

Chapter 5 demonstrates that sputum calprotectin is readily measurable by ELISA and that it can differentiate CF from control subjects with a similar statistical significance and sensitivity/specificity to more established biomarkers such as IL-8 and MPO. These findings suggest that calprotectin is readily measurable in sputum by a clinically relevant technique as compared to the highly sophisticated technique of mass spectrometry employed in the preceding chapters. Furthermore calprotectin is readily measurable in the serum of CF patients and differentiates them from control subjects in a similar manner to sputum calprotectin, thus providing an alternative means of measuring calprotectin in CF patients, which does not necessitate the sampling of sputum. Chapter 5 also demonstrate that sputum VEGF is lower in CF patients than control, an unexpected finding when one considers the previous findings of elevated levels of VEGF in CF serum(278).

8.1.3 The Measurement of Non-Protein Biomarkers in Sputum

Chapter 6 describes the measurement of metal ions in sputum. Previous studies have demonstrated raised iron levels in the sputum of CF patients(229, 230). Calprotectin may exert extracellular effects by chelation of zinc and thus the measurement of zinc levels in sputum seemed appropriate. Sputum zinc levels were indeed raised in sputum from patients with CF and bronchiectasis, and zinc and calprotectin were

highly correlated in the CF group. Sputum iron and copper are raised in CF, bronchiectasis and COPD sputum compared to control. The potential mechanistic interactions of calprotectin and zinc in the lung are of interest and merit further investigation.

8.1.4 Longitudinal Measurement of Biomarkers In Sputum and Serum in CF Patients

Chapter 7 assesses the usefulness, among other markers, of sputum and serum calprotectin in the monitoring of CF patients during an exacerbation of lung disease. Sputum and serum calprotectin decrease following treatment of an exacerbation, which is in keeping with changes in lung function in these patients, but not tracked by changes in IL-8 or MPO as might have been expected. Furthermore serum calprotectin levels may predict future outcome following an exacerbation in terms of time to next exacerbation. A change is also noted in sputum levels of zinc, a finding that matches that of sputum calprotectin, further underlining the potential use of zinc as a biomarker in CF and its possible mechanistic interaction with calprotectin.

8.2 Possible Insights Into The Pathophysiology of CF

The work in this thesis describes a number of biomarkers that are relevant to CF and possibly some other respiratory diseases. As well as serving as biomarkers they may also offer some insight into the disease process of CF, which is characterized by chronic lung infection and inflammation. Calprotectin has been previously described in CF but its role in the pathophysiology of the disease is poorly understood. There is now a growing interest in the potential pro- and anti-inflammatory effects of

calprotectin leading to some recent high profile research publications(268, 269). The data presented in this thesis demonstrates that it is not only present in CF but also that calgranulin A (a subunit of calprotectin) is present as a truncated peptide in CF and bronchiectatic sputum. This suggests a possible post-translational modification of calgranulin A or possibly degradation in situ in the chronically inflamed lung. This is of novel interest and should certainly form the basis of further work.

In this thesis I have also observed increased levels of zinc in CF sputum. As such this is the first described observation of this phenomenon. Calprotectin is a molecule with proposed zinc chelating properties and therefore the observation of calprotectin and zinc levels in sputum being closely correlated is of interest and may also be of mechanistic importance, which is further highlighted by recent findings in the literature(286).

8.3 Proposed Further Work

8.3.1 Calprotectin as Clinical Biomarker

Calprotectin is of potential use as a biomarker in CF. Further work is clearly indicated to validate the findings in both sputum and serum in CF patients. The measurement of calprotectin may also be relevant to other predominantly neutrophil mediated pathologies such as community acquired pneumonia. Of particular interest is the ability of serum calprotectin to predict outcome in CF. From the data presented in this thesis it is evident that patients with a lower serum calprotectin at the end of exacerbation fare better. I would propose a longitudinal study of stable CF patients to observe whether an increased calprotectin level at baseline predicts outcome over

a 1 year follow up period in terms of mortality and morbidity. Further to this, clinical studies to assess the effects of standard interventions in CF on serum and sputum calprotectin levels, such as macrolide anti-biotic therapy and nebulised DNAse may allow one insight into how interventions may effect the underlying pathophysiology of disease.

8.3.2 Calprotectin as a Biomarker in Gene Therapy Trials

The ultimate aim of this thesis was to discover new biomarkers for use in gene therapy trials as part of the UK CF Gene Therapy consortium (UKCFGTC). The evidence that I present to date demonstrates the worth of calprotectin and zinc measurements on outcomes in CF in cross sectional studies and longitudinally in exacerbation treated by antibiotic therapy. Sputum and serum calprotectin and sputum zinc certainly merit further investigation in CF and will be assessed in future gene therapy studies for CF by the UKCFGTC.

8.3.3 Calprotectin as a Key Mediator in CF Lung Inflammation

The link between the genetic mutation in CF and the onset of lung inflammation is poorly understood. Calprotectin is present in high concentrations in CF sputum and serum and may offer a unique insight into CF inflammation. Recent evidence has demonstrated calprotectin to be beneficial(286) and detrimental(268) in murine models of inflammation. Furthermore calprotectin has been demonstrated to have major effects via the activation of toll like receptor 4 (TLR-4)(269), a major pro-inflammatory pathway which is also stimulated by bacterial LPS, e.g. produced by *Pseudomonas aeruginosa* (PA) a major bacterial pathogen in CF. Therefore one

could propose that in the CF lung not only bacteria, but endogenous proteins may be stimulating major inflammatory pathways.

It has recently been proposed that PA infection in CF may stimulate a TH-17 response (a recently described form of inflammation in which Interleukin-17 is released as a key mediator) via interactions with TLR-4 on dendritic cells(332). This mechanism is proposed as a possible driver for inflammation in the CF lung, however not all patients with CF are chronically infected with PA, particularly not during childhood and therefore endogenous stimulation of this system by calprotectin as a TLR-4 ligand may be implicated. I would therefore propose investigation of the activation TH-17 responses via TLR-4 by calprotectin as an early driver of inflammation in CF lung disease. Studies will be required to focus on confirming activation of dendritic cells via TLR-4 *in vitro* by calprotectin and also measuring the response to calprotectin in in-vivo models.

The release of calprotectin from neutrophils is also of interest. Recently published data suggests that calprotectin is released by netrophils during death by NETosis(265), a recently described form of neutrophil cell death by which DNA and proteins are released into the surrounding area in web-like structures(333, 334). This would suggest that calprotectin may be present in the CF airway complexed to DNA and thus be a central component of the highly viscous mucous characteristic of the CF airway. Further work concerning the release of calprotectin and its potential role in inflammation in CF will therefore provide further information on the importance of this molecule.

8.4 Conclusions

This thesis has demonstrated the discovery and characterisation of biomarkers of CF lung disease in sputum. The most significant biomarker discovered by this process in sputum was calprotectin and the measurement of this molecule with immunoassay in sputum and serum is demonstrated, further underlining its potential as a biomarker. A relationship with the trace element zinc, in sputum, is also suggested. The usefulness of calprotectin measurement as a biomarker in CF was confirmed by its measurement in exacerbating CF patients before and after therapy, demonstrating significant changes in both sputum and serum measurements. Furthermore potential prognostic information was derived from the serum calprotectin levels following exacerbation. In conclusion the data in this thesis strongly endorses the use of sputum and serum calprotectin (among others) as non-invasive biomarkers of inflammation in CF. Further studies will be required to validate these findings in larger populations.

9.0 Bibliography

1. Boucher RC. New concepts of the pathogenesis of cystic fibrosis lung disease. Eur Respir J. 2004 January 1, 2004;23(1):146-58.
2. Rosenstein BJ, Zeitlin PL. Cystic fibrosis. Lancet. 1998 Jan 24;351(9098):277-82.
3. CF Foundation. Patient Registry 2006 Annual Report. 2008.
4. Morgan WJ, Butler SM, Johnson CA, Colin AA, FitzSimmons SC, Geller DE, et al. Epidemiologic study of cystic fibrosis: design and implementation of a prospective, multicenter, observational study of patients with cystic fibrosis in the U.S. and Canada. Pediatr Pulmonol. 1999 Oct;28(4):231-41.
5. Rochholz EL. Kindelied und Kinderspiel. 1857;280.
6. Andersen D. Cystic fibrosis of the pancreas and its relation to celiac disease: a clinical and pathological study. Am J Dis Child. 1938;56:344-99.
7. Quinton PM. Missing Cl conductance in cystic fibrosis. Am J Physiol. 1986 Oct;251(4 Pt 1):C649-52.
8. Knowles M, Gatzy J, Boucher R. Relative ion permeability of normal and cystic fibrosis nasal epithelium. J Clin Invest. 1983 May;71(5):1410-7.
9. Welsh MJ. An apical-membrane chloride channel in human tracheal epithelium. Science. 1986 Jun 27;232(4758):1648-50.
10. Welsh MJ, Liedtke CM. Chloride and potassium channels in cystic fibrosis airway epithelia. Nature. 1986 Jul 31-Aug 6;322(6078):467-70.
11. Schoumacher RA, Shoemaker RL, Halm DR, Tallant EA, Wallace RW, Frizzell RA. Phosphorylation fails to activate chloride channels from cystic fibrosis airway cells. Nature. 1987 Dec 24-31;330(6150):752-4.
12. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science. 1989 Sep 8;245(4922):1066-73.
13. Dorin JR, Novak M, Hill RE, Brock DJ, Secher DS, van Heyningen V. A clue to the basic defect in cystic fibrosis from cloning the CF antigen gene. Nature. 1987 Apr 9-15;326(6113):614-7.
14. Crystal RG, McElvaney NG, Rosenfeld MA, Chu CS, Mastrangeli A, Hay JG, et al. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. Nat Genet. 1994 Sep;8(1):42-51.
15. Boucher RC, Knowles MR, Johnson LG, Olsen JC, Pickles R, Wilson JM, et al. Gene therapy for cystic fibrosis using E1-deleted adenovirus: a phase I trial in the nasal cavity. The University of North Carolina at Chapel Hill. Hum Gene Ther. 1994 May;5(5):615-39.
16. Worldwide survey of the delta F508 mutation--report from the cystic fibrosis genetic analysis consortium. Am J Hum Genet. 1990 Aug;47(2):354-9.
17. Zielenski J. Genotype and phenotype in cystic fibrosis. Respiration. 2000;67(2):117-33.
18. Kulczycki LL, Kostuch M, Bellanti JA. A clinical perspective of cystic fibrosis and new genetic findings: relationship of CFTR mutations to genotype-phenotype manifestations. Am J Med Genet A. 2003 Jan 30;116A(3):262-7.
19. Hamosh A, Rosenstein BJ, Cutting GR. CFTR nonsense mutations G542X and W1282X associated with severe reduction of CFTR mRNA in nasal epithelial cells. Hum Mol Genet. 1992 Oct;1(7):542-4.

20. Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, et al. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell. 1990 Nov 16;63(4):827-34.
21. Welsh MJ, Smith AE. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell. 1993 Jul 2;73(7):1251-4.
22. Anderson MP, Welsh MJ. Regulation by ATP and ADP of CFTR chloride channels that contain mutant nucleotide-binding domains. Science. 1992 Sep 18;257(5077):1701-4.
23. Highsmith WE, Jr., Burch LH, Zhou Z, Olsen JC, Strong TV, Smith T, et al. Identification of a splice site mutation (2789 +5 G > A) associated with small amounts of normal CFTR mRNA and mild cystic fibrosis. Hum Mutat. 1997;9(4):332-8.
24. Kerem E, Corey M, Kerem BS, Rommens J, Markiewicz D, Levison H, et al. The relation between genotype and phenotype in cystic fibrosis--analysis of the most common mutation (delta F508). N Engl J Med. 1990 Nov 29;323(22):1517-22.
25. Anguiano A, Oates RD, Amos JA, Dean M, Gerrard B, Stewart C, et al. Congenital bilateral absence of the vas deferens. A primarily genital form of cystic fibrosis. JAMA. 1992 Apr 1;267(13):1794-7.
26. Ho LP, Samways JM, Porteous DJ, Dorin JR, Carothers A, Greening AP, et al. Correlation between nasal potential difference measurements, genotype and clinical condition in patients with cystic fibrosis. Eur Respir J. 1997 Sep;10(9):2018-22.
27. Drumm ML, Konstan MW, Schluchter MD, Handler A, Pace R, Zou F, et al. Genetic modifiers of lung disease in cystic fibrosis. N Engl J Med. 2005 Oct 6;353(14):1443-53.
28. Henry MT, Cave S, Rendall J, O'Connor CM, Morgan K, FitzGerald MX, et al. An alpha1-antitrypsin enhancer polymorphism is a genetic modifier of pulmonary outcome in cystic fibrosis. Eur J Hum Genet. 2001 Apr;9(4):273-8.
29. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, et al. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature. 1990 Jul 26;346(6282):362-5.
30. Welsh MJ, Smith AE. Cystic fibrosis. Sci Am. 1995 Dec;273(6):52-9.
31. Li M, McCann JD, Liedtke CM, Nairn AC, Greengard P, Welsh MJ. Cyclic AMP-dependent protein kinase opens chloride channels in normal but not cystic fibrosis airway epithelium. Nature. 1988 Jan 28;331(6154):358-60.
32. Hwang TC, Lu L, Zeitlin PL, Gruenert DC, Huganir R, Guggino WB. Cl-channels in CF: lack of activation by protein kinase C and cAMP-dependent protein kinase. Science. 1989 Jun 16;244(4910):1351-3.
33. Bear CE, Duguay F, Naismith AL, Kartner N, Hanrahan JW, Riordan JR. Cl-channel activity in Xenopus oocytes expressing the cystic fibrosis gene. J Biol Chem. 1991 Oct 15;266(29):19142-5.
34. Drumm ML, Wilkinson DJ, Smit LS, Worrell RT, Strong TV, Frizzell RA, et al. Chloride conductance expressed by delta F508 and other mutant CFTRs in Xenopus oocytes. Science. 1991 Dec 20;254(5039):1797-9.
35. Anderson MP, Rich DP, Gregory RJ, Smith AE, Welsh MJ. Generation of cAMP-activated chloride currents by expression of CFTR. Science. 1991 Feb 8;251(4994):679-82.

36. Drumm ML, Pope HA, Cliff WH, Rommens JM, Marvin SA, Tsui LC, et al. Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. Cell. 1990 Sep 21;62(6):1227-33.
37. Rich DP, Anderson MP, Gregory RJ, Cheng SH, Paul S, Jefferson DM, et al. Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. Nature. 1990 Sep 27;347(6291):358-63.
38. Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, et al. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. Science. 1991 Jul 12;253(5016):202-5.
39. Bear CE, Li CH, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, et al. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell. 1992 Feb 21;68(4):809-18.
40. Cheng SH, Rich DP, Marshall J, Gregory RJ, Welsh MJ, Smith AE. Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. Cell. 1991 Sep 6;66(5):1027-36.
41. Anderson MP, Berger HA, Rich DP, Gregory RJ, Smith AE, Welsh MJ. Nucleoside triphosphates are required to open the CFTR chloride channel. Cell. 1991 Nov 15;67(4):775-84.
42. Rich DP, Gregory RJ, Anderson MP, Manavalan P, Smith AE, Welsh MJ. Effect of deleting the R domain on CFTR-generated chloride channels. Science. 1991 Jul 12;253(5016):205-7.
43. Ma J, Tasch JE, Tao T, Zhao J, Xie J, Drumm ML, et al. Phosphorylation-dependent block of cystic fibrosis transmembrane conductance regulator chloride channel by exogenous R domain protein. J Biol Chem. 1996 Mar 29;271(13):7351-6.
44. Boucher RC, Stutts MJ, Knowles MR, Cantley L, Gatzy JT. Na+ transport in cystic fibrosis respiratory epithelia. Abnormal basal rate and response to adenylate cyclase activation. J Clin Invest. 1986 Nov;78(5):1245-52.
45. Johnson LG, Boyles SE, Wilson J, Boucher RC. Normalization of raised sodium absorption and raised calcium-mediated chloride secretion by adenovirus-mediated expression of cystic fibrosis transmembrane conductance regulator in primary human cystic fibrosis airway epithelial cells. J Clin Invest. 1995 Mar;95(3):1377-82.
46. Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, et al. CFTR as a cAMP-dependent regulator of sodium channels. Science. 1995 Aug 11;269(5225):847-50.
47. Ismailov, II, Awayda MS, Jovov B, Berdiev BK, Fuller CM, Dedman JR, et al. Regulation of epithelial sodium channels by the cystic fibrosis transmembrane conductance regulator. J Biol Chem. 1996 Mar 1;271(9):4725-32.
48. Stutts MJ, Rossier BC, Boucher RC. Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel single channel kinetics. J Biol Chem. 1997 May 30;272(22):14037-40.
49. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. Nat Med. 2004 May;10(5):487-93.

50. Gabriel SE, Clarke LL, Boucher RC, Stutts MJ. CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship. Nature. 1993 May 20;363(6426):263-8.
51. Grubb BR, Vick RN, Boucher RC. Hyperabsorption of Na+ and raised Ca(2+)-mediated Cl- secretion in nasal epithelia of CF mice. Am J Physiol. 1994 May;266(5 Pt 1):C1478-83.
52. Choi JY, Muallem D, Kiselyov K, Lee MG, Thomas PJ, Muallem S. Aberrant CFTR-dependent HCO3- transport in mutations associated with cystic fibrosis. Nature. 2001 Mar 1;410(6824):94-7.
53. McNicholas CM, Guggino WB, Schwiebert EM, Hebert SC, Giebisch G, Egan ME. Sensitivity of a renal K+ channel (ROMK2) to the inhibitory sulfonylurea compound glibenclamide is enhanced by coexpression with the ATP-binding cassette transporter cystic fibrosis transmembrane regulator. Proc Natl Acad Sci U S A. 1996 Jul 23;93(15):8083-8.
54. Pier GB, Grout M, Zaidi TS, Goldberg JB. How mutant CFTR may contribute to Pseudomonas aeruginosa infection in cystic fibrosis. Am J Respir Crit Care Med. 1996 Oct;154(4 Pt 2):S175-82.
55. Pier GB. Role of the cystic fibrosis transmembrane conductance regulator in innate immunity to Pseudomonas aeruginosa infections. Proc Natl Acad Sci U S A. 2000 Aug 1;97(16):8822-8.
56. Reiniger N, Lee MM, Coleman FT, Ray C, Golan DE, Pier GB. Resistance to Pseudomonas aeruginosa Chronic Lung Infection Requires Cystic Fibrosis Transmembrane Conductance Regulator-Modulated Interleukin-1 (IL-1) Release and Signaling through the IL-1 Receptor. Infect Immun. 2007 April 1, 2007;75(4):1598-608.
57. Quinton PM. Chloride impermeability in cystic fibrosis. Nature. 1983 Feb 3;301(5899):421-2.
58. Quinton PM, Bijman J. Higher bioelectric potentials due to decreased chloride absorption in the sweat glands of patients with cystic fibrosis. N Engl J Med. 1983 May 19;308(20):1185-9.
59. Reddy MM, Quinton PM. Altered electrical potential profile of human reabsorptive sweat duct cells in cystic fibrosis. Am J Physiol. 1989 Oct;257(4 Pt 1):C722-6.
60. Reddy MM, Quinton PM. Localization of Cl- conductance in normal and Cl-impermeability in cystic fibrosis sweat duct epithelium. Am J Physiol. 1989 Oct;257(4 Pt 1):C727-35.
61. Nussbaum E, Boat TF, Wood RE, Doershuk CF. Cystic fibrosis with acute hypoelectrolytemia and metabolic alkalosis in infancy. Am J Dis Child. 1979 Sep;133(9):965-6.
62. Di Sant'Agnese PA, Darling RC, Perera GA, Shea E. Abnormal electrolyte composition of sweat in cystic fibrosis of the pancreas; clinical significance and relationship to the disease. Pediatrics. 1953 Nov;12(5):549-63.
63. Durie PR. Pathophysiology of the pancreas in cystic fibrosis. Neth J Med. 1992 Oct;41(3-4):97-100.
64. Finkelstein SM, Wielinski CL, Elliott GR, Warwick WJ, Barbosa J, Wu SC, et al. Diabetes mellitus associated with cystic fibrosis. J Pediatr. 1988 Mar;112(3):373-7.
65. Ziegler MM. Meconium ileus. Curr Probl Surg. 1994 Sep;31(9):731-77.

66. Davidson DJ, Dorin JR. The CF mouse: an important tool for studying cystic fibrosis. Expert Rev Mol Med. 2001 Mar 12;2001:1-27.
67. O'Brien S, Keogan M, Casey M, Duffy G, McErlean D, Fitzgerald MX, et al. Biliary complications of cystic fibrosis. Gut. 1992 Mar;33(3):387-91.
68. Nash KL, Allison ME, McKeon D, Lomas DJ, Haworth CS, Bilton D, et al. A single centre experience of liver disease in adults with cystic fibrosis 1995-2006. J Cyst Fibros. 2008 May;7(3):252-7.
69. Seale TW, Flux M, Rennert OM. Reproductive defects in patients of both sexes with cystic fibrosis: a review. Ann Clin Lab Sci. 1985 Mar-Apr;15(2):152-8.
70. Sturgess J, Imrie J. Quantitative evaluation of the development of tracheal submucosal glands in infants with cystic fibrosis and control infants. Am J Pathol. 1982 Mar;106(3):303-11.
71. Esterly JR, Oppenheimer EH. Cystic fibrosis of the pancreas: structural changes in peripheral airways. Thorax. 1968 Nov;23(6):670-5.
72. Chow CW, Landau LI, Taussig LM. Bronchial mucous glands in the newborn with cystic fibrosis. Eur J Pediatr. 1982 Dec;139(4):240-3.
73. Wood RE, Boat TF, Doershuk CF. Cystic fibrosis. Am Rev Respir Dis. 1976 Jun;113(6):833-78.
74. Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DW. Early pulmonary inflammation in infants with cystic fibrosis. Am J Respir Crit Care Med. 1995 Apr;151(4):1075-82.
75. Cohen JC, Larson JE. Pathophysiologic consequences following inhibition of a CFTR-dependent developmental cascade in the lung. BMC Dev Biol. 2005;5:2.
76. Thomas GR, Costelloe EA, Lunn DP, Stacey KJ, Delaney SJ, Passey R, et al. G551D cystic fibrosis mice exhibit abnormal regulation of inflammation in lungs and macrophages. J Immunol. 2000 Apr 1;164(7):3870-7.
77. Donaldson SH, Corcoran TE, Laube BL, Bennett WD. Mucociliary clearance as an outcome measure for cystic fibrosis clinical research. Proc Am Thorac Soc. 2007 Aug 1;4(4):399-405.
78. Smith JJ, Travis SM, Greenberg EP, Welsh MJ. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell. 1996 Apr 19;85(2):229-36.
79. Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. Cell. 1997 Feb 21;88(4):553-60.
80. Zabner J, Smith JJ, Karp PH, Widdicombe JH, Welsh MJ. Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro. Mol Cell. 1998 Sep;2(3):397-403.
81. Knowles MR, Robinson JM, Wood RE, Pue CA, Mentz WM, Wager GC, et al. Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. J Clin Invest. 1997 Nov 15;100(10):2588-95.
82. Davies JC, Alton EW, Bush A. Cystic fibrosis. BMJ. 2007 Dec 15;335(7632):1255-9.
83. Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, et al. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. Cell. 1998 Dec 23;95(7):1005-15.

84. Grubb BR, Chadburn JL, Boucher RC. In vivo microdialysis for determination of nasal liquid ion composition. Am J Physiol Cell Physiol. 2002 Jun;282(6):C1423-31.
85. Hull J, Skinner W, Robertson C, Phelan P. Elemental content of airway surface liquid from infants with cystic fibrosis. Am J Respir Crit Care Med. 1998 Jan;157(1):10-4.
86. Jayaraman S, Song Y, Vetrivel L, Shankar L, Verkman AS. Noninvasive in vivo fluorescence measurement of airway-surface liquid depth, salt concentration, and pH. J Clin Invest. 2001 Feb;107(3):317-24.
87. Muhlebach MS, Stewart PW, Leigh MW, Noah TL. Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. Am J Respir Crit Care Med. 1999 Jul;160(1):186-91.
88. Armstrong DS, Grimwood K, Carlin JB, Carzino R, Gutierrez JP, Hull J, et al. Lower airway inflammation in infants and young children with cystic fibrosis. Am J Respir Crit Care Med. 1997 Oct;156(4 Pt 1):1197-204.
89. Rosenfeld M, Gibson RL, McNamara S, Emerson J, Burns JL, Castile R, et al. Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. Pediatr Pulmonol. 2001 Nov;32(5):356-66.
90. Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. Am J Respir Crit Care Med. 1999 Nov;160(5 Pt 2):S5-11.
91. Watt AP, Courtney J, Moore J, Ennis M, Elborn JS. Neutrophil cell death, activation and bacterial infection in cystic fibrosis. Thorax. 2005 Aug;60(8):659-64.
92. Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, et al. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. J Clin Invest. 2002 Mar;109(5):661-70.
93. Banner KH, De Jonge H, Elborn S, Growcott E, Gulbins E, Konstan M, et al. Highlights of a workshop to discuss targeting inflammation in cystic fibrosis. Journal of Cystic Fibrosis. 2009;8(1):1-8.
94. Demko CA, Byard PJ, Davis PB. Gender differences in cystic fibrosis: Pseudomonas aeruginosa infection. J Clin Epidemiol. 1995 Aug;48(8):1041-9.
95. Pedersen SS, Hoiby N, Espersen F, Koch C. Role of alginate in infection with mucoid Pseudomonas aeruginosa in cystic fibrosis. Thorax. 1992 Jan;47(1):6-13.
96. Parad RB, Gerard CJ, Zurakowski D, Nichols DP, Pier GB. Pulmonary outcome in cystic fibrosis is influenced primarily by mucoid Pseudomonas aeruginosa infection and immune status and only modestly by genotype. Infect Immun. 1999 Sep;67(9):4744-50.
97. Campodonico VL, Gadjeva M, Paradis-Bleau C, Uluer A, Pier GB. Airway epithelial control of Pseudomonas aeruginosa infection in cystic fibrosis. Trends Mol Med. 2008 Mar;14(3):120-33.
98. Goss CH, Burns JL. Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. Thorax. 2007 Apr;62(4):360-7.
99. Smith AL, Redding G, Doershuk C, Goldmann D, Gore E, Hilman B, et al. Sputum changes associated with therapy for endobronchial exacerbation in cystic fibrosis. J Pediatr. 1988 Apr;112(4):547-54.
100. Aaron SD, Ramotar K, Ferris W, Vandemheen K, Saginur R, Tullis E, et al. Adult cystic fibrosis exacerbations and new strains of Pseudomonas aeruginosa. Am J Respir Crit Care Med. 2004 Apr 1;169(7):811-5.

101. Hiatt PW, Grace SC, Kozinetz CA, Raboudi SH, Treece DG, Taber LH, et al. Effects of viral lower respiratory tract infection on lung function in infants with cystic fibrosis. Pediatrics. 1999 Mar;103(3):619-26.
102. Ordonez CL, Henig NR, Mayer-Hamblett N, Accurso FJ, Burns JL, Chmiel JF, et al. Inflammatory and microbiologic markers in induced sputum after intravenous antibiotics in cystic fibrosis. Am J Respir Crit Care Med. 2003 Dec 15;168(12):1471-5.
103. Colombo C, Costantini D, Rocchi A, Cariani L, Garlaschi ML, Tirelli S, et al. Cytokine levels in sputum of cystic fibrosis patients before and after antibiotic therapy. Pediatr Pulmonol. 2005 Jul;40(1):15-21.
104. Ramsey BW, Pepe MS, Quan JM, Otto KL, Montgomery AB, Williams-Warren J, et al. Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. Cystic Fibrosis Inhaled Tobramycin Study Group. N Engl J Med. 1999 Jan 7;340(1):23-30.
105. Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, et al. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. N Engl J Med. 1994 Sep 8;331(10):637-42.
106. MacLusky IB, Gold R, Corey M, Levison H. Long-term effects of inhaled tobramycin in patients with cystic fibrosis colonized with Pseudomonas aeruginosa. Pediatr Pulmonol. 1989;7(1):42-8.
107. Ramsey BW, Dorkin HL, Eisenberg JD, Gibson RL, Harwood IR, Kravitz RM, et al. Efficacy of aerosolized tobramycin in patients with cystic fibrosis. N Engl J Med. 1993 Jun 17;328(24):1740-6.
108. Ryan G, Mukhopadhyay S, Singh M. Nebulised anti-pseudomonal antibiotics for cystic fibrosis. Cochrane Database Syst Rev. 2003(3):CD001021.
109. Flume PA, O'Sullivan BP, Robinson KA, Goss CH, Mogayzel PJ, Jr., Willey-Courand DB, et al. Cystic fibrosis pulmonary guidelines: chronic medications for maintenance of lung health. Am J Respir Crit Care Med. 2007 Nov 15;176(10):957-69.
110. McCoy K, Hamilton S, Johnson C. Effects of 12-week administration of dornase alfa in patients with advanced cystic fibrosis lung disease. Pulmozyme Study Group. Chest. 1996 Oct;110(4):889-95.
111. Jones AP, Wallis CE. Recombinant human deoxyribonuclease for cystic fibrosis. Cochrane Database Syst Rev. 2003(3):CD001127.
112. Quan JM, Tiddens HA, Sy JP, McKenzie SG, Montgomery MD, Robinson PJ, et al. A two-year randomized, placebo-controlled trial of dornase alfa in young patients with cystic fibrosis with mild lung function abnormalities. J Pediatr. 2001 Dec;139(6):813-20.
113. Donaldson SH, Bennett WD, Zeman KL, Knowles MR, Tarran R, Boucher RC. Mucus clearance and lung function in cystic fibrosis with hypertonic saline. N Engl J Med. 2006 Jan 19;354(3):241-50.
114. Eng PA, Morton J, Douglass JA, Riedler J, Wilson J, Robertson CF. Short-term efficacy of ultrasonically nebulized hypertonic saline in cystic fibrosis. Pediatr Pulmonol. 1996 Feb;21(2):77-83.
115. Elkins MR, Robinson M, Rose BR, Harbour C, Moriarty CP, Marks GB, et al. A controlled trial of long-term inhaled hypertonic saline in patients with cystic fibrosis. N Engl J Med. 2006 Jan 19;354(3):229-40.

116. Minasian C, Wallis C, Metcalfe C, Bush A. Comparison of inhaled mannitol, daily rhDNase and a combination of both in children with cystic fibrosis: a randomised trial. Thorax. January 2010;65(1):51-6.
117. Balfour-Lynn IM, Lees B, Hall P, Phillips G, Khan M, Flather M, et al. Multicenter randomized controlled trial of withdrawal of inhaled corticosteroids in cystic fibrosis. Am J Respir Crit Care Med. 2006 Jun 15;173(12):1356-62.
118. Eigen H, Rosenstein BJ, FitzSimmons S, Schidlow DV. A multicenter study of alternate-day prednisone therapy in patients with cystic fibrosis. Cystic Fibrosis Foundation Prednisone Trial Group. J Pediatr. 1995 Apr;126(4):515-23.
119. Cheng K, Ashby D, Smyth R. Oral steroids for cystic fibrosis. Cochrane Database Syst Rev. 2000(2):CD000407.
120. Konstan MW, Byard PJ, Hoppel CL, Davis PB. Effect of high-dose ibuprofen in patients with cystic fibrosis. N Engl J Med. 1995 Mar 30;332(13):848-54.
121. Koyama H, Geddes DM. Erythromycin and diffuse panbronchiolitis. Thorax. 1997 Oct;52(10):915-8.
122. Saiman L, Marshall BC, Mayer-Hamblett N, Burns JL, Quittner AL, Cibene DA, et al. Azithromycin in patients with cystic fibrosis chronically infected with Pseudomonas aeruginosa: a randomized controlled trial. JAMA. 2003 Oct 1;290(13):1749-56.
123. Wolter J, Seeney S, Bell S, Bowler S, Masel P, McCormack J. Effect of long term treatment with azithromycin on disease parameters in cystic fibrosis: a randomised trial. Thorax. 2002 Mar;57(3):212-6.
124. Equi A, Balfour-Lynn IM, Bush A, Rosenthal M. Long term azithromycin in children with cystic fibrosis: a randomised, placebo-controlled crossover trial. Lancet. 2002 Sep 28;360(9338):978-84.
125. Dakin C, Henry RL, Field P, Morton J. Defining an exacerbation of pulmonary disease in cystic fibrosis. Pediatr Pulmonol. 2001 Jun;31(6):436-42.
126. Smyth A, Elborn JS. Exacerbations in cystic fibrosis: 3--Management. Thorax. 2008 Feb;63(2):180-4.
127. Cheng K, Smyth RL, Govan JR, Doherty C, Winstanley C, Denning N, et al. Spread of beta-lactam-resistant Pseudomonas aeruginosa in a cystic fibrosis clinic. Lancet. 1996 Sep 7;348(9028):639-42.
128. Elphick HE, Tan A. Single versus combination intravenous antibiotic therapy for people with cystic fibrosis. Cochrane Database Syst Rev. 2005(2):CD002007.
129. Aaron SD, Vandemheen KL, Ferris W, Fergusson D, Tullis E, Haase D, et al. Combination antibiotic susceptibility testing to treat exacerbations of cystic fibrosis associated with multiresistant bacteria: a randomised, double-blind, controlled clinical trial. Lancet. 2005 Aug 6-12;366(9484):463-71.
130. Glanville AR, Estenne M. Indications, patient selection and timing of referral for lung transplantation. Eur Respir J. 2003 November 1, 2003;22(5):845-52.
131. Meachery G, De Soyza A, Nicholson A, Parry G, Hasan A, Tocewicz K, et al. Outcomes of lung transplantation for cystic fibrosis in a large UK cohort. Thorax. 2008 August 2008;63(8):725-31.
132. Goldman MJ, Yang Y, Wilson JM. Gene therapy in a xenograft model of cystic fibrosis lung corrects chloride transport more effectively than the sodium defect. Nat Genet. 1995 Feb;9(2):126-31.
133. Griesenbach U, Geddes DM, Alton EW. Update on gene therapy for cystic fibrosis. Curr Opin Mol Ther. 2003 Oct;5(5):489-94.

134. Ferrari S, Griesenbach U, Geddes DM, Alton E. Immunological hurdles to lung gene therapy. Clin Exp Immunol. 2003 Apr;132(1):1-8.
135. Harvey BG, Leopold PL, Hackett NR, Grasso TM, Williams PM, Tucker AL, et al. Airway epithelial CFTR mRNA expression in cystic fibrosis patients after repetitive administration of a recombinant adenovirus. J Clin Invest. 1999 Nov;104(9):1245-55.
136. Kobinger GP, Weiner DJ, Yu QC, Wilson JM. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. Nat Biotechnol. 2001 Mar;19(3):225-30.
137. Alton EW, Stern M, Farley R, Jaffe A, Chadwick SL, Phillips J, et al. Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. Lancet. 1999 Mar 20;353(9157):947-54.
138. Hyde SC, Southern KW, Gileadi U, Fitzjohn EM, Mofford KA, Waddell BE, et al. Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. Gene Ther. 2000 Jul;7(13):1156-65.
139. Porteous DJ, Dorin JR, McLachlan G, Davidson-Smith H, Davidson H, Stevenson BJ, et al. Evidence for safety and efficacy of DOTAP cationic liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Gene Ther. 1997 Mar;4(3):210-8.
140. McLachlan G, Baker A, Tennant P, Gordon C, Vrettou C, Renwick L, et al. Optimizing aerosol gene delivery and expression in the ovine lung. Mol Ther. 2007 Feb;15(2):348-54.
141. Hirsh AJ, Molino BF, Zhang J, Astakhova N, Geiss WB, Sargent BJ, et al. Design, synthesis, and structure-activity relationships of novel 2-substituted pyrazinoylguanidine epithelial sodium channel blockers: drugs for cystic fibrosis and chronic bronchitis. J Med Chem. 2006 Jul 13;49(14):4098-115.
142. Kunzelmann K, Mall M. Pharmacotherapy of the ion transport defect in cystic fibrosis: role of purinergic receptor agonists and other potential therapeutics. Am J Respir Med. 2003;2(4):299-309.
143. Deterding RR, Lavange LM, Engels JM, Mathews DW, Coquillette SJ, Brody AS, et al. Phase 2 randomized safety and efficacy trial of nebulized denufosol tetrasodium in cystic fibrosis. Am J Respir Crit Care Med. 2007 Aug 15;176(4):362-9.
144. Wilschanski M, Yahav Y, Yaacov Y, Blau H, Bentur L, Rivlin J, et al. Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. N Engl J Med. 2003 Oct 9;349(15):1433-41.
145. Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, et al. PTC124 targets genetic disorders caused by nonsense mutations. Nature. 2007 May 3;447(7140):87-91.
146. Hirawat S, Welch EM, Elfring GL, Northcutt VJ, Paushkin S, Hwang S, et al. Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers. J Clin Pharmacol. 2007 Apr;47(4):430-44.
147. Kerem E, Hirawat S, Armoni S, Yaakov Y, Shoseyov D, Cohen M, et al. Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial. The Lancet.372(9640):719-27.

148. Rubenstein RC, Zeitlin PL. A pilot clinical trial of oral sodium 4-phenylbutyrate (Buphenyl) in deltaF508-homozygous cystic fibrosis patients: partial restoration of nasal epithelial CFTR function. Am J Respir Crit Care Med. 1998 Feb;157(2):484-90.
149. Schluchter MD, Konstan MW, Drumm ML, Yankaskas JR, Knowles MR. Classifying Severity of Cystic Fibrosis Lung Disease Using Longitudinal Pulmonary Function Data. Am J Respir Crit Care Med. 2006 October 1, 2006;174(7):780-6.
150. Kerem E, Reisman J, Corey M, Canny GJ, Levison H. Prediction of mortality in patients with cystic fibrosis. N Engl J Med. 1992 Apr 30;326(18):1187-91.
151. Corey M, Farewell V. Determinants of mortality from cystic fibrosis in Canada, 1970-1989. Am J Epidemiol. 1996 May 15;143(10):1007-17.
152. Mayer-Hamblett N, Rosenfeld M, Emerson J, Goss CH, Aitken ML. Developing cystic fibrosis lung transplant referral criteria using predictors of 2-year mortality. Am J Respir Crit Care Med. 2002 Dec 15;166(12 Pt 1):1550-5.
153. Davis PB, Byard PJ, Konstan MW. Identifying treatments that halt progression of pulmonary disease in cystic fibrosis. Pediatr Res. 1997 Feb;41(2):161-5.
154. Horsley AR, Gustafsson PM, Macleod KA, Saunders C, Greening AP, Porteous DJ, et al. Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis. Thorax. 2008 Feb;63(2):135-40.
155. Downing G, editor. NIH Definitions Working Group. Biomarkers and surrogate endpoints in clinical research: definitions and conceptual model.: Elselvier ; 2000.
156. Bonfield TL, Panuska JR, Konstan MW, Hilliard KA, Hilliard JB, Ghnaim H, et al. Inflammatory cytokines in cystic fibrosis lungs [published erratum appears in Am J Respir Crit Care Med 1996 Oct;154(4 Pt 1):following 1217]. Am J Respir Crit Care Med. 1995 December 1, 1995;152(6):2111-8.
157. Tate S, MacGregor G, Davis M, Innes JA, Greening AP. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. Thorax. 2002 Nov;57(11):926-9.
158. Ho LP, Innes JA, Greening AP. Nitrite levels in breath condensate of patients with cystic fibrosis is elevated in contrast to exhaled nitric oxide. Thorax. 1998 Aug;53(8):680-4.
159. Bonfield TL, Konstan MW, Burfeind P, Panuska JR, Hilliard JB, Berger M. Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis. Am J Respir Cell Mol Biol. 1995 Sep;13(3):257-61.
160. Konstan MW, Walenga RW, Hilliard KA, Hilliard JB. Leukotriene B4 markedly elevated in the epithelial lining fluid of patients with cystic fibrosis. Am Rev Respir Dis. 1993 Oct;148(4 Pt 1):896-901.
161. Meyer KC, Zimmerman J. Neutrophil mediators, Pseudomonas, and pulmonary dysfunction in cystic fibrosis. J Lab Clin Med. 1993 May;121(5):654-61.
162. Birrer P, McElvaney NG, Rudeberg A, Sommer CW, Liechti-Gallati S, Kraemer R, et al. Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. Am J Respir Crit Care Med. 1994 Jul;150(1):207-13.
163. Konstan MW, Hilliard KA, Norvell TM, Berger M. Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest

ongoing infection and inflammation. Am J Respir Crit Care Med. 1994 Aug;150(2):448-54.
164. Noah TL, Black HR, Cheng PW, Wood RE, Leigh MW. Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. J Infect Dis. 1997 Mar;175(3):638-47.
165. Meyer KC, Lewandoski JR, Zimmerman JJ, Nunley D, Calhoun WJ, Dopico GA. Human neutrophil elastase and elastase/alpha 1-antiprotease complex in cystic fibrosis. Comparison with interstitial lung disease and evaluation of the effect of intravenously administered antibiotic therapy. Am Rev Respir Dis. 1991 Sep;144(3 Pt 1):580-5.
166. McElvaney NG, Hubbard RC, Birrer P, Chernick MS, Caplan DB, Frank MM, et al. Aerosol alpha 1-antitrypsin treatment for cystic fibrosis. Lancet. 1991 Feb 16;337(8738):392-4.
167. McElvaney NG, Nakamura H, Birrer P, Hebert CA, Wong WL, Alphonso M, et al. Modulation of airway inflammation in cystic fibrosis. In vivo suppression of interleukin-8 levels on the respiratory epithelial surface by aerosolization of recombinant secretory leukoprotease inhibitor. J Clin Invest. 1992 Oct;90(4):1296-301.
168. Wagener JS, Rock MJ, McCubbin MM, Hamilton SD, Johnson CA, Ahrens RC. Aerosol delivery and safety of recombinant human deoxyribonuclease in young children with cystic fibrosis: a bronchoscopic study. Pulmozyme Pediatric Broncoscopy Study Group. J Pediatr. 1998 Oct;133(4):486-91.
169. Roum JH, Borok Z, McElvaney NG, Grimes GJ, Bokser AD, Buhl R, et al. Glutathione aerosol suppresses lung epithelial surface inflammatory cell-derived oxidants in cystic fibrosis. J Appl Physiol. 1999 Jul;87(1):438-43.
170. Rosenfeld M, Gibson R, McNamara S, Emerson J, McCoyd KS, Shell R, et al. Serum and lower respiratory tract drug concentrations after tobramycin inhalation in young children with cystic fibrosis. J Pediatr. 2001 Oct;139(4):572-7.
171. Gibson RL, Emerson J, McNamara S, Burns JL, Rosenfeld M, Yunker A, et al. Significant microbiological effect of inhaled tobramycin in young children with cystic fibrosis. Am J Respir Crit Care Med. 2003 Mar 15;167(6):841-9.
172. Ratjen F, Paul K, van Koningsbruggen S, Breitenstein S, Rietschel E, Nikolaizik W. DNA concentrations in BAL fluid of cystic fibrosis patients with early lung disease: influence of treatment with dornase alpha. Pediatr Pulmonol. 2005 Jan;39(1):1-4.
173. Sagel SD, Chmiel JF, Konstan MW. Sputum Biomarkers of Inflammation in Cystic Fibrosis Lung Disease. Proc Am Thorac Soc. 2007 August 1, 2007;4(4):406-17.
174. Macgregor G, Gray RD, Hilliard TN, Imrie M, Boyd AC, Alton EW, et al. Biomarkers for cystic fibrosis lung disease: Application of SELDI-TOF mass spectrometry to BAL fluid. J Cyst Fibros. 2008 Sep;7(5):352-8.
175. McMorran BJ, Patat SA, Carlin JB, Grimwood K, Jones A, Armstrong DS, et al. Novel neutrophil-derived proteins in bronchoalveolar lavage fluid indicate an exaggerated inflammatory response in pediatric cystic fibrosis patients. Clin Chem. 2007 Oct;53(10):1782-91.
176. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. Lancet. 2002 Nov 30;360(9347):1715-21.

177. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax. 2002 Oct;57(10):875-9.
178. Hargreave FE, Leigh R. Induced sputum, eosinophilic bronchitis, and chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 1999 Nov;160(5 Pt 2):S53-7.
179. Pavord ID, Pizzichini MM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. Thorax. 1997 Jun;52(6):498-501.
180. Pizzichini MM, Popov TA, Efthimiadis A, Hussack P, Evans S, Pizzichini E, et al. Spontaneous and induced sputum to measure indices of airway inflammation in asthma. Am J Respir Crit Care Med. 1996 Oct;154(4 Pt 1):866-9.
181. Belda J, Leigh R, Parameswaran K, O'Byrne PM, Sears MR, Hargreave FE. Induced sputum cell counts in healthy adults. Am J Respir Crit Care Med. 2000 Feb;161(2 Pt 1):475-8.
182. Spanevello A, Confalonieri M, Sulotto F, Romano F, Balzano G, Migliori GB, et al. Induced sputum cellularity. Reference values and distribution in normal volunteers. Am J Respir Crit Care Med. 2000 Sep;162(3 Pt 1):1172-4.
183. Sagel SD, Accurso FJ. Monitoring inflammation in CF. Cytokines. Clin Rev Allergy Immunol. 2002 Aug;23(1):41-57.
184. Dean TP, Dai Y, Shute JK, Church MK, Warner JO. Interleukin-8 concentrations are elevated in bronchoalveolar lavage, sputum, and sera of children with cystic fibrosis. Pediatr Res. 1993 Aug;34(2):159-61.
185. Kronborg G, Hansen MB, Svenson M, Fomsgaard A, Hoiby N, Bendtzen K. Cytokines in sputum and serum from patients with cystic fibrosis and chronic Pseudomonas aeruginosa infection as markers of destructive inflammation in the lungs. Pediatr Pulmonol. 1993 May;15(5):292-7.
186. Bruce MC, Poncz L, Klinger JD, Stern RC, Tomashefski JF, Jr., Dearborn DG. Biochemical and pathologic evidence for proteolytic destruction of lung connective tissue in cystic fibrosis. Am Rev Respir Dis. 1985 Sep;132(3):529-35.
187. Richman-Eisenstat JB, Jorens PG, Hebert CA, Ueki I, Nadel JA. Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. Am J Physiol. 1993 Apr;264(4 Pt 1):L413-8.
188. Greally P, Hussein MJ, Cook AJ, Sampson AP, Piper PJ, Price JF. Sputum tumour necrosis factor-alpha and leukotriene concentrations in cystic fibrosis. Arch Dis Child. 1993 Mar;68(3):389-92.
189. Regelmann WE, Siefferman CM, Herron JM, Elliott GR, Clawson CC, Gray BH. Sputum peroxidase activity correlates with the severity of lung disease in cystic fibrosis. Pediatr Pulmonol. 1995 Jan;19(1):1-9.
190. Wolter JM, Rodwell RL, Bowler SD, McCormack JG. Cytokines and inflammatory mediators do not indicate acute infection in cystic fibrosis. Clin Diagn Lab Immunol. 1999 Mar;6(2):260-5.
191. Pukhalsky AL, Kapranov NI, Kalashnikova EA, Shmarina GV, Shabalova LA, Kokarovtseva SN, et al. Inflammatory markers in cystic fibrosis patients with lung Pseudomonas aeruginosa infection. Mediators Inflamm. 1999;8(3):159-67.
192. Karpati F, Hjelte FL, Wretlind B. TNF-alpha and IL-8 in consecutive sputum samples from cystic fibrosis patients during antibiotic treatment. Scand J Infect Dis. 2000;32(1):75-9.

193. Mayer-Hamblett N, Aitken ML, Accurso FJ, Kronmal RA, Konstan MW, Burns JL, et al. Association between Pulmonary Function and Sputum Biomarkers in Cystic Fibrosis. Am J Respir Crit Care Med. 2007 Apr 15;175(8):822-8.
194. Ordonez CL, Kartashov AI, Wohl ME. Variability of markers of inflammation and infection in induced sputum in children with cystic fibrosis. J Pediatr. 2004 Nov;145(5):689-92.
195. Ordonez CL, Stulbarg M, Grundland H, Liu JT, Boushey HA. Effect of clarithromycin on airway obstruction and inflammatory markers in induced sputum in cystic fibrosis: a pilot study. Pediatr Pulmonol. 2001 Jul;32(1):29-37.
196. Pedersen SK, Sloane AJ, Prasad SS, Sebastian LT, Lindner RA, Hsu M, et al. An immunoproteomic approach for identification of clinical biomarkers for monitoring disease: application to cystic fibrosis. Molecular & Cellular Proteomics 4(8):1052-60, 2005 Aug.
197. Sagel SD, Kapsner R, Osberg I, Sontag MK, Accurso FJ. Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. Am J Respir Crit Care Med. 2001 Oct 15;164(8 Pt 1):1425-31.
198. Sagel SD, Kapsner RK, Osberg I. Induced sputum matrix metalloproteinase-9 correlates with lung function and airway inflammation in children with cystic fibrosis. Pediatr Pulmonol. 2005 Mar;39(3):224-32.
199. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. J Pediatr. 2002 Dec;141(6):811-7.
200. Gilljam H, Malmborg AS, Strandvik B. Conformity of bacterial growth in sputum and contamination free endobronchial samples in patients with cystic fibrosis. Thorax. 1986 Aug;41(8):641-6.
201. Thomassen MJ, Klinger JD, Badger SJ, van Heeckeren DW, Stern RC. Cultures of thoracotomy specimens confirm usefulness of sputum cultures in cystic fibrosis. J Pediatr. 1984 Mar;104(3):352-6.
202. Aaron SD, Kottachchi D, Ferris WJ, Vandemheen KL, St Denis ML, Plouffe A, et al. Sputum versus bronchoscopy for diagnosis of Pseudomonas aeruginosa biofilms in cystic fibrosis. Eur Respir J. 2004 Oct;24(4):631-7.
203. Kolak M, Karpati F, Monstein HJ, Jonasson J. Molecular typing of the bacterial flora in sputum of cystic fibrosis patients. Int J Med Microbiol. 2003 Aug;293(4):309-17.
204. Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 2003 Aug;41(8):3548-58.
205. Kim JS, Okamoto K, Rubin BK. Pulmonary function is negatively correlated with sputum inflammatory markers and cough clearability in subjects with cystic fibrosis but not those with chronic bronchitis. Chest. 2006 May;129(5):1148-54.
206. Djukanovic R, Sterk PJ, Fahy JV, Hargreave{section} FE. Standardised methodology of sputum induction and processing 10.1183/09031936.02.00000102. Eur Respir J. 2002 July 1, 2002;20(37_suppl):1S-2.
207. M.M. Kelly, Keatings V, Leigh R, Peterson C, Shute J, Venge P, et al. Analysis of fluid{-}phase mediators 10.1183/09031936.02.00002402. Eur Respir J. 2002 July 1, 2002;20(37_suppl):24S-39.

208. Sloane AJ, Lindner RA, Prasad SS, Sebastian LT, Pedersen SK, Robinson M, et al. Proteomic analysis of sputum from adults and children with cystic fibrosis and from control subjects. Am J Respir Crit Care Med. 2005 Dec 1;172(11):1416-26.
209. Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988 Oct 15;60(20):2299-301.
210. T. William Hutchens T-TY. New desorption strategies for the mass spectrometric analysis of macromolecules. Rapid Communications in Mass Spectrometry. 1993;7(7):576-80.
211. Uchida T, Fukawa A, Uchida M, Fujita K, Saito K. Application of a novel protein biochip technology for detection and identification of rheumatoid arthritis biomarkers in synovial fluid. J Proteome Res. 2002 Nov-Dec;1(6):495-9.
212. Zhang L, Yu W, He T, Yu J, Caffrey RE, Dalmasso EA, et al. Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. Science. 2002 Nov 1;298(5595):995-1000.
213. Xiao Z, Adam BL, Cazares LH, Clements MA, Davis JW, Schellhammer PF, et al. Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. Cancer Res. 2001 Aug 15;61(16):6029-33.
214. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, et al. Use of proteomic patterns in serum to identify ovarian cancer. Lancet. 2002 Feb 16;359(9306):572-7.
215. Katherine R. Kozak FS, Julian P. Whitelegge, Kym Faull, Srinivasa Reddy, Robin Farias-Eisner,. Characterization of serum biomarkers for detection of early stage ovarian cancer. PROTEOMICS. 2005;5(17):4589-96.
216. Ranganathan S, Williams E, Ganchev P, Gopalakrishnan V, Lacomis D, Urbinelli L, et al. Proteomic profiling of cerebrospinal fluid identifies biomarkers for amyotrophic lateral sclerosis. J Neurochem. 2005 Dec;95(5):1461-71.
217. Estelle Peronnet LB, Florence Poirier, Myriam Cubizolles, Geneviève Choquet-Kastylevsky, Colette Jolivet-Reynaud,. SELDI-TOF MS analysis of the Cardiac Troponin I forms present in plasma from patients with myocardial infarction. PROTEOMICS. 2006;6(23):6288-99.
218. Agranoff D, Fernandez-Reyes D, Papadopoulos MC, Rojas SA, Herbster M, Loosemore A, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. Lancet. 2006 Sep 16;368(9540):1012-21.
219. Papadopoulos MC, Abel PM, Agranoff D, Stich A, Tarelli E, Bell BA, et al. A novel and accurate diagnostic test for human African trypanosomiasis. Lancet. 2004 Apr 24;363(9418):1358-63.
220. Abramovitz M, Leyland-Jones B. A systems approach to clinical oncology: focus on breast cancer. Proteome Sci. 2006;4:5.
221. Baggerly KA, Morris JS, Coombes KR. Reproducibility of SELDI-TOF protein patterns in serum: comparing datasets from different experiments. Bioinformatics. 2004 Mar 22;20(5):777-85.
222. White MY, Gundry RL, Cordwell SJ. When does a fingerprint constitute a diagnostic?[comment]. Lancet 368(9540):971-3, 2006 Sep 16.
223. Merkel D, Rist W, Seither P, Weith A, Lenter MC. Proteomic study of human bronchoalveolar lavage fluids from smokers with chronic obstructive pulmonary disease by combining surface-enhanced laser desorption/ionization-mass

spectrometry profiling with mass spectrometric protein identification. Proteomics. 2005 Jul;5(11):2972-80.
224. Kriegova E, Melle C, Kolek V, Hutyrova B, Mrazek F, Bleul A, et al. Protein Profiles of Bronchoalveolar Lavage Fluid from Patients with Pulmonary Sarcoidosis 10.1164/rccm.200507-1126OC. Am J Respir Crit Care Med. 2006 May 15, 2006;173(10):1145-54.
225. Nicholas B, Skipp P, Mould R, Rennard S, Davies DE, O'Connor CD, et al. Shotgun proteomic analysis of human-induced sputum. Proteomics 6(15):4390-401, 2006 Aug.
226. Henkin JA, Jennings ME, Matthews DE, Vigoreaux JO. Mass processing--an improved technique for protein identification with mass spectrometry data. J Biomol Tech. 2004 Dec;15(4):230-7.
227. Stites SW, Walters B, O'Brien-Ladner AR, Bailey K, Wesselius LJ. Increased iron and ferritin content of sputum from patients with cystic fibrosis or chronic bronchitis. Chest. 1998 Sep;114(3):814-9.
228. Stites SW, Plautz MW, Bailey K, O'Brien-Ladner AR, Wesselius LJ. Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with stable cystic fibrosis. Am J Respir Crit Care Med. 1999 Sep;160(3):796-801.
229. Reid DW, Withers NJ, Francis L, Wilson JW, Kotsimbos TC. Iron deficiency in cystic fibrosis: relationship to lung disease severity and chronic Pseudomonas aeruginosa infection. Chest. 2002 Jan;121(1):48-54.
230. Reid DW, Lam QT, Schneider H, Walters EH. Airway iron and iron-regulatory cytokines in cystic fibrosis. Eur Respir J. 2004 Aug;24(2):286-91.
231. BTS. British Guideline on the Management of Asthma. Thorax. 2003;58 (suppl 1):i1-i83.
232. BTS. Chronic Obstructive Pulmonary Disease. National clinical guideline on management of chronic obstructive pulmonary disease in adults in primary and secondary care. Thorax. 2004;59(suppl1):i1-i232.
233. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J. 2005 Aug;26(2):319-38.
234. Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P. Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int. 2004 Jan;65(1):323-32.
235. Schipper R, Loof A, de Groot J, Harthoorn L, Dransfield E, van Heerde W. SELDI-TOF-MS of saliva: methodology and pre-treatment effects. J Chromatogr B Analyt Technol Biomed Life Sci. 2007 Feb 15;847(1):45-53.
236. Smith AD, Cowan JO, Brassett KP, Herbison GP, Taylor DR. Use of exhaled nitric oxide measurements to guide treatment in chronic asthma. N Engl J Med. 2005 May 26;352(21):2163-73.
237. Smith AD, Cowan JO, Brassett KP, Filsell S, McLachlan C, Monti-Sheehan G, et al. Exhaled nitric oxide: a predictor of steroid response. Am J Respir Crit Care Med. 2005 Aug 15;172(4):453-9.
238. Kharitonov SA, Barnes PJ. Clinical aspects of exhaled nitric oxide. Eur Respir J. 2000 Oct;16(4):781-92.
239. Montuschi P, Kharitonov SA, Barnes PJ. Exhaled carbon monoxide and nitric oxide in COPD. Chest. 2001 Aug;120(2):496-501.

240. Kharitonov SA, Gonio F, Kelly C, Meah S, Barnes PJ. Reproducibility of exhaled nitric oxide measurements in healthy and asthmatic adults and children. Eur Respir J. 2003 Mar;21(3):433-8.
241. Stirling RG, Kharitonov SA, Campbell D, Robinson DS, Durham SR, Chung KF, et al. Increase in exhaled nitric oxide levels in patients with difficult asthma and correlation with symptoms and disease severity despite treatment with oral and inhaled corticosteroids. Asthma and Allergy Group. Thorax. 1998 Dec;53(12):1030-4.
242. Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. Lancet. 1994 Jan 15;343(8890):133-5.
243. Hunt J. Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease. J Allergy Clin Immunol. 2002 Jul;110(1):28-34.
244. Balint B, Kharitonov SA, Hanazawa T, Donnelly LE, Shah PL, Hodson ME, et al. Increased nitrotyrosine in exhaled breath condensate in cystic fibrosis. Eur Respir J. 2001 Jun;17(6):1201-7.
245. Hanazawa T, Kharitonov SA, Barnes PJ. Increased nitrotyrosine in exhaled breath condensate of patients with asthma. Am J Respir Crit Care Med. 2000 Oct;162(4 Pt 1):1273-6.
246. Montuschi P, Barnes PJ. Analysis of exhaled breath condensate for monitoring airway inflammation. Trends Pharmacol Sci. 2002 May;23(5):232-7.
247. Kharitonov SA, Barnes PJ. Biomarkers of some pulmonary diseases in exhaled breath. Biomarkers. 2002 Jan-Feb;7(1):1-32.
248. Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Baraldi E, et al. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J. 2005 Sep;26(3):523-48.
249. Ho LP, Faccenda J, Innes JA, Greening AP. Expired hydrogen peroxide in breath condensate of cystic fibrosis patients. Eur Respir J. 1999 Jan;13(1):103-6.
250. Alexis NE, Hu SC, Zeman K, Alter T, Bennett WD. Induced sputum derives from the central airways: confirmation using a radiolabeled aerosol bolus delivery technique. Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 1):1964-70.
251. Moodley YP, Krishnan V, Lalloo UG. Neutrophils in induced sputum arise from central airways. Eur Respir J. 2000 Jan;15(1):36-40.
252. Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. Am J Respir Crit Care Med. 1996 Aug;154(2 Pt 1):308-17.
253. Brightling CE. Clinical applications of induced sputum. Chest. 2006 May;129(5):1344-8.
254. Cooper GL, Louie A, Baltch AL, Chu RC, Smith RP, Ritz WJ, et al. Influence of zinc on Pseudomonas aeruginosa susceptibilities to imipenem. J Clin Microbiol. 1993 Sep;31(9):2366-70.
255. McGarvey LP, Dunbar K, Martin SL, Brown V, Macmahon J, Ennis M, et al. Cytokine concentrations and neutrophil elastase activity in bronchoalveolar lavage and induced sputum from patients with cystic fibrosis, mild asthma and healthy volunteers. J Cyst Fibros. 2002 Dec;1(4):269-75.

256. Reid DW, Misso N, Aggarwal S, Thompson PJ, Walters EH. Oxidative stress and lipid-derived inflammatory mediators during acute exacerbations of cystic fibrosis. Respirology. 2007 Jan;12(1):63-9.
257. Sagel SD, Dowell EB, Accurso FJ. Bacterial colonization and infection in the CF lung. Methods Mol Med. 2002;70:433-46.
258. Downey DG, Martin SL, Dempster M, Moore JE, Keogan MT, Starcher B, et al. The relationship of clinical and inflammatory markers to outcome in stable patients with cystic fibrosis. Pediatr Pulmonol. 2007 Mar;42(3):216-20.
259. Salva PS, Doyle NA, Graham L, Eigen H, Doerschuk CM. TNF-alpha, IL-8, soluble ICAM-1, and neutrophils in sputum of cystic fibrosis patients. Pediatr Pulmonol. 1996 Jan;21(1):11-9.
260. Sagel SD. Identifying novel endpoints for cystic fibrosis clinical trials. Adv Pediatr. 2005;52:115-27.
261. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, et al. Use of induced sputum cell counts to investigate airway inflammation in asthma. Thorax. 1992 Jan;47(1):25-9.
262. Sinha S, Watorek W, Karr S, Giles J, Bode W, Travis J. Primary structure of human neutrophil elastase. Proc Natl Acad Sci U S A. 1987 Apr;84(8):2228-32.
263. van Heyningen V, Dorin J. Possible role for two calcium-binding proteins of the S-100 family, co-expressed in granulocytes and certain epithelia. Adv Exp Med Biol. 1990;269:139-43.
264. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. [Review] [48 refs]. Journal of Leukocyte Biology 53(2):197-204, 1993 Feb.
265. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil Extracellular Traps Contain Calprotectin, a Cytosolic Protein Complex Involved in Host Defense against Candida albicans. PLoS Pathog. 2009;5(10):e1000639.
266. Schulze zur Wiesch A, Foell D, Frosch M, Vogl T, Sorg C, Roth J. Myeloid related proteins MRP8/MRP14 may predict disease flares in juvenile idiopathic arthritis. Clin Exp Rheumatol. 2004 May-Jun;22(3):368-73.
267. Silberer H, Kuppers B, Mickisch O, Baniewicz W, Drescher M, Traber L, et al. Fecal leukocyte proteins in inflammatory bowel disease and irritable bowel syndrome. Clin Lab. 2005;51(3-4):117-26.
268. Raquil MA, Anceriz N, Rouleau P, Tessier PA. Blockade of antimicrobial proteins S100A8 and S100A9 inhibits phagocyte migration to the alveoli in streptococcal pneumonia. J Immunol. 2008 Mar 1;180(5):3366-74.
269. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007 Sep;13(9):1042-9.
270. Shijubo N, Itoh Y, Yamaguchi T, Sugaya F, Hirasawa M, Yamada T, et al. Serum levels of Clara cell 10-kDa protein are decreased in patients with asthma. Lung. 1999;177(1):45-52.
271. Johansson S, Keen C, Stahl A, Wennergren G, Benson M. Low levels of CC16 in nasal fluid of children with birch pollen-induced rhinitis. Allergy. 2005 May;60(5):638-42.

272. Kettle AJ, Chan T, Osberg I, Senthilmohan R, Chapman ALP, Mocatta TJ, et al. Myeloperoxidase and Protein Oxidation in the Airways of Young Children with Cystic Fibrosis
10.1164/rccm.200311-1516OC. Am J Respir Crit Care Med. 2004 December 15, 2004;170(12):1317-23.
273. Stockley RA, Dale I, Hill SL, Fagerhol MK. Relationship of neutrophil cytoplasmic protein (L1) to acute and chronic lung disease. Scand J Clin Lab Invest. 1984 Nov;44(7):629-34.
274. Striz I, Trebichavsky I. Calprotectin - a pleiotropic molecule in acute and chronic inflammation. Physiol Res. 2004;53(3):245-53.
275. Starosta V, Ratjen F, Rietschel E, Paul K, Griese M. Anti-inflammatory cytokines in cystic fibrosis lung disease. Eur Respir J. 2006 September 1, 2006;28(3):581-7.
276. Arsalane K, Broeckaert F, Knoops B, Wiedig M, Toubeau G, Bernard A. Clara Cell Specific Protein (CC16) Expression after Acute Lung Inflammation Induced by Intratracheal Lipopolysaccharide Administration. Am J Respir Crit Care Med. 2000 May 1, 2000;161(5):1624-30.
277. Meyer KC. Neutrophils, myeloperoxidase, and bronchiectasis in cystic fibrosis: green is not good. J Lab Clin Med. 2004 Sep;144(3):124-6.
278. McColley SA, Stellmach V, Boas SR, Jain M, Crawford SE. Serum vascular endothelial growth factor is elevated in cystic fibrosis and decreases with treatment of acute pulmonary exacerbation. Am J Respir Crit Care Med. 2000 Jun;161(6):1877-80.
279. Rabin HR, Butler SM, Wohl ME, Geller DE, Colin AA, Schidlow DV, et al. Pulmonary exacerbations in cystic fibrosis. Pediatr Pulmonol. 2004 May;37(5):400-6.
280. Henig NR, Tonelli MR, Pier MV, Burns JL, Aitken ML. Sputum induction as a research tool for sampling the airways of subjects with cystic fibrosis. Thorax. 2001 Apr;56(4):306-11.
281. Pham I, Uchida T, Planes C, Ware LB, Kaner R, Matthay MA, et al. Hypoxia upregulates VEGF expression in alveolar epithelial cells in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol. 2002 November 1, 2002;283(5):L1133-42.
282. Wilson GB, Fudenberg HH, Jahn TL. Studies on cystic fibrosis using isoelectric focusing. I. An assay for detection of cystic fibrosis homozygotes and heterozygote carriers from serum. Pediatric Research. 1975 August 1975;9(8):635-40.
283. Krebs NF, Westcott JE, Arnold TD, Kluger BM, Accurso FJ, Miller LV, et al. Abnormalities in zinc homeostasis in young infants with cystic fibrosis. Pediatr Res. 2000 Aug;48(2):256-61.
284. Mutti A, Corradi M, Goldoni M, Vettori MV, Bernard A, Apostoli P. Exhaled Metallic Elements and Serum Pneumoproteins in Asymptomatic Smokers and Patients With COPD or Asthma*. Chest. 2006 May 2006;129(5):1288-97.
285. Bryant RE, Crouse R, Deagen JT. Zinc, iron, copper, selenium, lactoferrin, and ferritin in human pus. Am J Med Sci. 2004 Feb;327(2):73-6.
286. Corbin BD, Seeley EH, Raab A, Feldmann J, Miller MR, Torres VJ, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. Science. 2008 Feb 15;319(5865):962-5.

287. Calhoun NR, Smith JC. Uptake of 65Zn in fractured bones. Lancet. 1968 Sep 21;2(7569):682.
288. Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. Am J Respir Crit Care Med. 2003 Jun 15;167(12):1600-19.
289. Zalewski PD, Truong-Tran AQ, Grosser D, Jayaram L, Murgia C, Ruffin RE. Zinc metabolism in airway epithelium and airway inflammation: basic mechanisms and clinical targets. A review. Pharmacol Ther. 2005 Feb;105(2):127-49.
290. Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. Nature. 2002 May 30;417(6888):552-5.
291. Olson JC, Ohman DE. Efficient production and processing of elastase and LasA by Pseudomonas aeruginosa require zinc and calcium ions. J Bacteriol. 1992 Jun;174(12):4140-7.
292. Brumlik MJ, Storey DG. Zinc and iron regulate translation of the gene encoding Pseudomonas aeruginosa elastase. Mol Microbiol. 1992 Feb;6(3):337-44.
293. Zalewski PD. Zinc metabolism in the airway: basic mechanisms and drug targets. Curr Opin Pharmacol. 2006 Jun;6(3):237-43.
294. Ho LH, Ruffin RE, Murgia C, Li L, Krilis SA, Zalewski PD. Labile zinc and zinc transporter ZnT4 in mast cell granules: role in regulation of caspase activation and NF-kappaB translocation. J Immunol. 2004 Jun 15;172(12):7750-60.
295. Truong-Tran AQ, Ruffin RE, Foster PS, Koskinen AM, Coyle P, Philcox JC, et al. Altered zinc homeostasis and caspase-3 activity in murine allergic airway inflammation. Am J Respir Cell Mol Biol. 2002 Sep;27(3):286-96.
296. Gomez NN, Davicino RC, Biaggio VS, Bianco GA, Alvarez SM, Fischer P, et al. Overexpression of inducible nitric oxide synthase and cyclooxygenase-2 in rat zinc-deficient lung: Involvement of a NF-kappaB dependent pathway. Nitric Oxide. 2006 Feb;14(1):30-8.
297. Maret W. Zinc coordination environments in proteins determine zinc functions. J Trace Elem Med Biol. 2005;19(1):7-12.
298. Frederickson CJ. Neurobiology of zinc and zinc-containing neurons. Int Rev Neurobiol. 1989;31:145-238.
299. Truong-Tran AQ, Ruffin RE, Zalewski PD. Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines. Am J Physiol Lung Cell Mol Physiol. 2000 Dec;279(6):L1172-83.
300. Truong-Tran AQ, Grosser D, Ruffin RE, Murgia C, Zalewski PD. Apoptosis in the normal and inflamed airway epithelium: role of zinc in epithelial protection and procaspase-3 regulation. Biochem Pharmacol. 2003 Oct 15;66(8):1459-68.
301. Carter JE, Truong-Tran AQ, Grosser D, Ho L, Ruffin RE, Zalewski PD. Involvement of redox events in caspase activation in zinc-depleted airway epithelial cells. Biochem Biophys Res Commun. 2002 Oct 4;297(4):1062-70.
302. Jurado RL. Iron, infections, and anemia of inflammation. Clin Infect Dis. 1997 Oct;25(4):888-95.
303. Lusitani D, Malawista SE, Montgomery RR. Calprotectin, an abundant cytosolic protein from human polymorphonuclear leukocytes, inhibits the growth of Borrelia burgdorferi. Infect Immun. 2003 Aug;71(8):4711-6.
304. Clohessy PA, Golden BE. Calprotectin-mediated zinc chelation as a biostatic mechanism in host defence. Scand J Immunol. 1995 Nov;42(5):551-6.

305. M.M. Kelly, Keatings V, Leigh R, Peterson C, Shute J, Venge P, et al. Analysis of fluid phase mediators. Eur Respir J. 2002 July 1, 2002;20(37_suppl):24S-39.
306. Beeley JM, Darke CS, Owen GCR. Serum zinc, bronchiectasis, and bronchial carcinoma. Thorax. 1974 Jan;29(1):21-5.
307. Andrews GS. Studies of plasma zinc, copper, caeruloplasmin, and growth hormone: with special reference to carcinoma of the bronchus. J Clin Pathol. 1979 Apr;32(4):325-33.
308. Greaves MW, Dawber R. Zinc in psoriasis. Lancet. 1970 Jun 13;1(7659):1295.
309. Greaves MW, Skillen AW. Effects of long-continued ingestion of zinc sulphate in patients with venous leg ulceration. Lancet. 1970 Oct 31;2(7679):889-91.
310. Chang KL, Hung TC, Hsieh BS, Chen YH, Chen TF, Cheng HL. Zinc at pharmacologic concentrations affects cytokine expression and induces apoptosis of human peripheral blood mononuclear cells. Nutrition. 2006 Feb 9.
311. Driessen C, Hirv K, Rink L, Kirchner H. Induction of cytokines by zinc ions in human peripheral blood mononuclear cells and separated monocytes. Lymphokine Cytokine Res. 1994 Feb;13(1):15-20.
312. Wellinghausen N, Schromm AB, Seydel U, Brandenburg K, Luhm J, Kirchner H, et al. Zinc enhances lipopolysaccharide-induced monokine secretion by alteration of fluidity state of lipopolysaccharide. J Immunol. 1996 Oct 1;157(7):3139-45.
313. Ibs KH, Rink L. Zinc-altered immune function. J Nutr. 2003 May;133(5 Suppl 1):1452S-6S.
314. Bao S, Knoell DL. Zinc modulates airway epithelium susceptibility to death receptor-mediated apoptosis. Am J Physiol Lung Cell Mol Physiol. 2006 March 1, 2006;290(3):L433-41.
315. Zsembery A, Fortenberry JA, Liang L, Bebok Z, Tucker TA, Boyce AT, et al. Extracellular zinc and ATP restore chloride secretion across cystic fibrosis airway epithelia by triggering calcium entry. J Biol Chem. 2004 Mar 12;279(11):10720-9.
316. Zalewski P, Truong-Tran A, Lincoln S, Ward D, Shankar A, Coyle P, et al. Use of a zinc fluorophore to measure labile pools of zinc in body fluids and cell-conditioned media. Biotechniques. 2006 Apr;40(4):509-20.
317. Nelson ME, O'Brien-Ladner AR, Wesselius LJ. Regional variation in iron and iron-binding proteins within the lungs of smokers. Am J Respir Crit Care Med. 1996 Apr;153(4 Pt 1):1353-8.
318. Isaksen B, Fagerhol MK. Calprotectin inhibits matrix metalloproteinases by sequestration of zinc. Mol Pathol. 2001 October 1, 2001;54(5):289-92.
319. Ghavami S, Kerkhoff C, Los M, Hashemi M, Sorg C, Karami-Tehrani F. Mechanism of apoptosis induced by S100A8/A9 in colon cancer cell lines: the role of ROS and the effect of metal ions. J Leukoc Biol. 2004 Jul;76(1):169-75.
320. Yui S, Nakatani Y, Hunter MJ, Chazin WJ, Yamazaki M. Implication of extracellular zinc exclusion by recombinant human calprotectin (MRP8 and MRP14) from target cells in its apoptosis-inducing activity. Mediators Inflamm. 2002 Jun;11(3):165-72.
321. Milne DB, Ralston NV, Wallwork JC. Zinc content of cellular components of blood: methods for cell separation and analysis evaluated. Clin Chem. 1985 Jan;31(1):65-9.

322. Voganatsi A, Panyutich A, Miyasaki KT, Murthy RK. Mechanism of extracellular release of human neutrophil calprotectin complex. J Leukoc Biol. 2001 Jul;70(1):130-4.
323. Marshall BC. Pulmonary exacerbations in cystic fibrosis: it's time to be explicit! Am J Respir Crit Care Med. 2004 Apr 1;169(7):781-2.
324. Redding GJ, Restuccia R, Cotton EK, Brooks JG. Serial changes in pulmonary functions in children hospitalized with cystic fibrosis. Am Rev Respir Dis. 1982 Jul;126(1):31-6.
325. Regelmann WE, Elliott GR, Warwick WJ, Clawson CC. Reduction of sputum Pseudomonas aeruginosa density by antibiotics improves lung function in cystic fibrosis more than do bronchodilators and chest physiotherapy alone. Am Rev Respir Dis. 1990 Apr;141(4 Pt 1):914-21.
326. Boussac M, Garin J. Calcium-dependent secretion in human neutrophils: a proteomic approach. Electrophoresis. 2000 Feb;21(3):665-72.
327. Husson MO, Wizla-Derambure N, Turck D, Gosset P, Wallaert B. Effect of intermittent inhaled tobramycin on sputum cytokine profiles in cystic fibrosis. J Antimicrob Chemother. 2005 Jul;56(1):247-9.
328. Downey DG, Brockbank S, Martin SL, Ennis M, Elborn JS. The effect of treatment of cystic fibrosis pulmonary exacerbations on airways and systemic inflammation. Pediatr Pulmonol. 2007 Aug 1;42(8):729-35.
329. Bland JM, Altman DG. Statistics notes: Multiple significance tests: the Bonferroni method. BMJ. 1995 January 21, 1995;310(6973):170.
330. Matsuyama W, Hashiguchi T, Matsumuro K, Iwami F, Hirotsu Y, Kawabata M, et al. Increased serum level of vascular endothelial growth factor in pulmonary tuberculosis. Am J Respir Crit Care Med. 2000 Sep;162(3 Pt 1):1120-2.
331. Schulz R, Hummel C, Heinemann S, Seeger W, Grimminger F. Serum levels of vascular endothelial growth factor are elevated in patients with obstructive sleep apnea and severe nighttime hypoxia. Am J Respir Crit Care Med. 2002 Jan 1;165(1):67-70.
332. Dubin PJ, McAllister F, Kolls JK. Is cystic fibrosis a TH17 disease? Inflamm Res. 2007 Jun 1;56(6):221-7.
333. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004 Mar 5;303(5663):1532-5.
334. Beneficial suicide: why neutrophils die to make NETs. Science (2007).

10.0 Publications Arising From This Thesis

1. **RD Gray**, G MacGregor, D Noble, M Imrie, M Dewar, AC Boyd, JA Innes, DJ Porteous, AP Greening. Sputum Proteomocs in Inflammatory and Suppurative Lung Disease (Original research article). Am J Respir Crit Care Med. 2008 Sep 1;178(5):444-52
2. **RD Gray**, A Duncan, D Noble, M Imrie, D St J O'Reilly, JA Innes, D Porteous, AP Greening, AC Boyd. Sputum Trace Metals Are Biomarkers of Disease Activity in Inflammatory and Suppurative Lung Diseases. Chest. 2010 March; 137(3):635-641
3. **RD Gray**, M Imrie, AC Boyd, JA Innes, DJ Porteous, AP Greening. The monitoring of Cystic Fibrosis Exacerbations with Sputum and Serum Biomarkers. Journal of Cystic Fibrosis 2010; 9: 193-198

Sputum Proteomics in Inflammatory and Suppurative Respiratory Diseases

Robert D. Gray[1], Gordon MacGregor[1], Donald Noble[1], Margaret Imrie[1], Maria Dewar[1], A. Christopher Boyd[1], J. Alastair Innes[1], David J. Porteous[1], and Andrew P. Greening[1]

[1]School of Molecular and Clinical Medicine, University of Edinburgh, Western General Hospital, Edinburgh, United Kingdom

Rationale: Markers of inflammatory activity are important for assessment and management of many respiratory diseases. Markers that are currently unrecognized may be more valuable than those presently believed to be useful.

Objectives: To identify potential biomarkers of suppurative and inflammatory lung disease in induced sputum samples.

Methods: Induced sputum was collected from 20 healthy control subjects, 24 patients with asthma, 24 with chronic obstructive pulmonary disease, 28 with cystic fibrosis (CF), and 19 with bronchiectasis. Twelve patients with CF had sputum sampled before and after antibiotic therapy for an infective exacerbation. The fluid phase of induced sputum was analyzed by surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectroscopy on three protein array surfaces. Some protein markers were selected for identification, and relevant ELISA assays sought. For 12 patients with CF, both SELDI-TOF and ELISA monitored changes in inflammatory responses during infective exacerbations.

Measurements and Main Results: SELDI-TOF identified potential biomarkers that differentiated each of the disease groups from healthy control subjects: at a significance of $P < 0.01$, there were 105 for asthma, 113 for chronic obstructive pulmonary disease, 381 for CF, and 377 for bronchiectasis. Peaks selected for protein identification yielded calgranulin A, calgranulin B, calgranulin C, Clara cell secretory protein, lysosyme c, proline rich salivary peptide, cystatin s, and hemoglobin α. On treatment of an infective CF exacerbation, SELDI-TOF determined falls in levels of calgranulin A and calgranulin B that were mirrored by ELISA-measured falls in calprotectin (heterodimer of calgranulins A and B).

Conclusions: Proteomic screening of sputum yields potential biomarkers of inflammation. The early development of a clinically relevant assay from such data is demonstrated.

Keywords: biomarkers; calprotectin; cystic fibrosis

Although there is considerable clinical and research need for good assessment of inflammation in airway diseases, the requirement of relatively invasive procedures, such as bronchoscopy and bronchoalveolar lavage (BAL), precludes sampling from a wide range of patients and on repeated occasions. This has encouraged investigators to use techniques of exhaled gases and exhaled breath condensate (EBC) analysis, although there may be limitations of value (1–10). The cellular phase of induced sputum is helpful in evaluating and monitoring asthma (11), but its use in suppurative lung diseases such as cystic fibrosis (CF) is less clear, although in children induced sputum measurements have been shown to correlate with lung function (12). The fluid phase of sputum has been rather underutilized; however, potential problems of variability in noncellular material may be encountered and as yet there is no agreed-upon correction factor as in BAL sampling.

Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) technology has been used to discover biomarkers of disease groups in direct comparison to control groups. The primary focus of much of this research has been to determine diagnostic biomarkers in a wide range of diseases, such as rheumatoid arthritis, HIV infection (13), prostate cancer (14), ovarian cancer (15, 16), motor neurone disease (17), ischemic heart disease (18), and infectious diseases (19, 20), using a diverse range of body fluids, such as serum, urine, cerebrospinal fluid, and joint fluid. The generated protein profiles demonstrate differences between disease groups and individual proteins may be identified to act as biomarkers either alone or in tandem with others. SELDI-TOF data may be used to generate serum "protein fingerprints" using bioinformatics to provide a potential diagnostic test in cancer and infectious diseases (15, 19, 20). This approach relies on interpretation of mass spectral patterns rather than identifying individual proteins and has been widely criticized in the literature and, in some cases, such as the work of Petricoin and colleagues (15), analysis of data sets independently have led to completely different conclusions (21). Baggerly and coworkers (21) demonstrated that inconsistent data processing can alter the ultimate outcome of these sorts of experiments. A different, and more direct, approach is to identify the actual proteins responsible for the mass spectral peaks provided by mass spectrometry (MS) data. Indeed, the identification of key biomarkers after initial proteomic studies may provide a more viable route to clinical application (22) and furthermore may allow the functional importance of such markers to be investigated. Therefore, using SELDI-TOF as a screening tool

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Induced sputum cytology has been used to investigate a number of respiratory diseases, including asthma. The fluid phase is less well characterized and likely to contain undiscovered biomarkers that may be used to monitor disease.

What This Study Adds to the Field

High-throughput mass spectrometry identifies key biomarkers in sputum, allowing quantitative measurement with immunoassay, and potential development of new clinically applicable tests of inflammation in lung disease.

(Received in original form March 13, 2007; accepted in final form June 17, 2008)

G.M. and A.C.B. were funded through the U.K. Cystic Fibrosis Gene Therapy Consortium. R.D.G. was supported by the Medical Research Council (program grant G9313618). D.N. was supported by an unrestricted Investigator Grant from GlaxoSmithKline.

Correspondence and requests for reprints should be addressed to Professor Andrew P. Greening, M.D., F.R.C.P., Respiratory Unit Offices, 1st Floor, Anne Ferguson Building, Western General Hospital, Crewe Road, South Edinburgh EH4 2XU, UK. E-mail: a.greening@ed.ac.uk

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 178. pp 444–452, 2008
Originally Published in Press as DOI: 10.1164/rccm.200703-409OC on June 19, 2008
Internet address: www.atsjournals.org

to identify individual potential biomarkers for further characterization may be a more valid and robust approach. SELDI-TOF has been applied to a small number of patients with chronic obstructive pulmonary disease (COPD) demonstrating a protein profile in BAL (23), and in pulmonary sarcoidosis (24). SELDI-TOF has been used to demonstrate protein expression in BAL in CF (25, 26). There is as yet a lack of published data using SELDI-TOF to profile sputum, although complementary proteomic approaches of two-dimensional electrophoresis (27), shotgun protein sequencing (28), and affinity immunoproteomics (29) have been applied to sputum in an effort to discover new biomarkers.

We applied SELDI-TOF to induced sputum from patients with inflammatory (asthma, COPD) and suppurative (CF, bronchiectasis) airway diseases to determine whether a high-throughput proteomics screening method can reveal differential protein expression between disease groups and therefore candidate biomarkers, which may then be developed into point assays of inflammation with potential application in the clinic. This work was driven by the need to develop biomarkers to monitor suppurative lung disease, in particular CF.

METHODS

Subjects and Selection

The Lothian Ethics Committee (Edinburgh, UK) granted ethical approval and all participants gave written consent. Sputum was obtained from 28 patients with CF, 19 with bronchiectasis, 24 with asthma, 24 with COPD, and 20 healthy control subjects. All patients were clinically stable and recruited from the outpatient clinic of a respiratory medicine unit. All subjects were recruited from specialist respiratory clinics at a major teaching hospital where they were attending with a diagnosis of their respective illness. In the absence of preexisting data on intersubject variability, formal statistical powering was not possible, but we approached this problem by recruiting similar numbers of subjects to previous biomarker studies. The patient groups represented respiratory diseases with clinical features that both complement and contrast with each other.

Longitudinal Data

To determine the usefulness of discovered markers in the assessment of lung disease during a changing level of inflammation, longitudinal samples were collected from 12 patients with CF during an infective exacerbation. Samples were collected at onset and completion of intravenous antibiotic therapy (duration, 14–21 d). The biomarkers were assessed both by SELDI-TOF and by ELISA.

Induced Sputum

Sputum induction was performed as previously described (30). Subjects inhaled nebulized hypertonic saline at concentrations of 3, 4, and 5%. Sputum was processed within 2 hours of collection (31). Sputum plugs were selected and processed with 4 × weight/volume of 0.1% dithiothreitol after which 4 × weight/volume of phosphate-buffered saline was added. Samples were filtered through 48-μm mesh and centrifuged to remove the cells. Supernatants were stored at −80°C until further analysis. Cytospins were stained with May-Grunwald-Giemsa for differential cell counting.

SELDI-TOF MS

Three different chromatographic chip surfaces were used to cover a wide range of protein characteristics: a weak cation exchange at pH 4 (CM10), an anion exchange at pH 10 (Q10), and an immobilized metal affinity surface activated with nickel (IMAC nickel [IMACNi]). Induced sputum samples were adjusted to contain 1 mg/ml protein at a concentration of 0.1% dithiothreitol. Ten microliters of sample were added to CM10 and Q10 surfaces in a bioprocessor unit (Ciphergen, Freemont, CA) and 1 μl of sample was added to the preactivated IMACNi surface for on-spot incubation (more reproducible for IMACNi surfaces). All chips were treated with sinapinic acid (SPA) matrix (2 × 0.8 μl/spot) and allowed to air dry. Samples were analyzed on the Protein Biology System 2 SELDI-TOF mass spectrometer (Ciphergen). Chips were read with a laser intensity of 205 with the deflector set at 4,000 D and a focus mass of 7,500 D. Profiles were then exported to Ciphergen Express (Ciphergen) for clustering and data analysis. Intra- and interassay coefficients of variation were assessed with the CM10 surface as outlined in the online supplement.

Data Analysis

Data were normalized to total ion current to take into account any spot to spot variability in chip surface binding. Data with high normalization coefficients were identified and individual spectra examined. Poor-quality spectra or absent signal were excluded from further analysis. Protein peaks were automatically clustered to identify biomarkers of similar molecular weight. Mann-Whitney rank testing was performed to demonstrate statistical differences in clusters between disease groups. Values of $P < 0.01$ were taken to be significant. For longitudinal data, paired t test analysis was performed using Prism 4 software (GraphPad, San Diego, CA). For a further description of methods, please refer to the online supplement.

Protein Identification

Highly significant peaks ($P < 0.0001$), assessed on relative abundance and quality of original spectral data, were selected for protein identification. Pooled sputum was used. Samples were applied to 18% trisene/glycine acrylimide gel for electrophoresis. Bands of appropriate molecular weight were excised and destained and the protein eluted. The eluate was digested with proteomics grade trypsin (Sigma, Gillingham, UK), applied to a normal-phase chromatography chip surface and analyzed with SELDI-TOF MS or MALDI (matrix-assisted laser desorption/ionization)-TOF MS using a Voyager DE STR mass spectrometer (Applied Biosystems, Foster City, CA). The resultant mass fingerprint was used to identify the parent protein through reference to protein identification databases (MS-Fit; University of California, San Francisco, San Francisco, CA). Proteins were identified based on molecular weight search (MOWSE) score and cross-checked with online MASCOT (http://www.matrixscience.com), which allows a probability-based MOWSE score. Protein identification was only accepted when P values were less than 0.05. Tandem MS/MS analysis was performed to confirm identification of proteins using a Q-star tandem MS instrument (Applied Biosystems) with SELDI chip interface (Ciphergen). This approach provided additional protein identification based on amino acid sequence from the MS/MS analysis.

Commercial antibodies, when available, were used for protein confirmation by Western blotting. For calgranulins A and B, we were also able to use an in-house quantitative immunoassay measuring calprotectin (calgranulin A/B complex). An in-house double antibody sandwich ELISA (antibodies and calprotectin standard courtesy of E. Sundrehagen, M.D.) was developed for use in the 12 patients with CF monitored longitudinally, and results compared with data from SELDI-TOF.

RESULTS

Demographics

Patient demographics are given in Table 1. Of the patients with CF, 18 were chronically infected with *Pseudomonas aeruginosa*.

Reproducibility of SELDI-TOF Assay in Sputum

This was assessed as described in the online supplement. The intraassay coefficient of variation ranged from 11.5 to 44% with an average coefficient of variation (CV) of 22.4%. Interassay variation demonstrated a CV ranging from 6 to 43%, with an average CV of 16.2%.

Protein Profiles Generated by SELDI-TOF

An example of generated data is demonstrated in Figures 1 and 2. Cluster analyses determined a large number of peaks present in

TABLE 1. DEMOGRAPHIC, LUNG FUNCTION, AND SPUTUM NEUTROPHIL DATA OF THE PATIENTS STUDIED

Group	Age (*yr*)	Sex	FEV_1 (*% pred*)	Sputum Neutrophil (%)
Asthma	47.8 (2.9)	15 F/8 M	82.3 (4.4)	50.6 (5.0)
COPD	65.2 (1.2)	10 F/14 M	57.7 (4.1)	79.1 (2.7)
Bronchiectasis	61.8 (3.5)	15 F/3 M	71.7 (11.6)	83.2 (5.6)
CF	28.8 (1.7)	8 F/19 M	59.2 (3.9)	91.6 (2.0)
Control	36.4 (2.1)	11 F/9 M	101.4 (3.1)	54.2 (5.3)

Definition of abbreviations: CF = cystic fibrosis; COPD = chronic obstructive pulmonary disease; F = females; M = males.
Values are mean (SEM).

relative proportions that differentiated between disease groups and healthy controls (Table 2). Three chip surfaces yielded a total of 621 clustered protein peaks, 105 of those significantly different at $P < 0.01$ and 56 at $P < 0.001$ for asthma versus healthy controls. For COPD compared with healthy controls, the respective figures were 625 protein peaks, with 113 significant at $P < 0.01$ and 50 at $P < 0.001$ for three surfaces. For bronchiectasis versus healthy controls, the respective figures were 671 protein peaks, with 377 significant at $P < 0.01$ and 314 at $P < 0.001$ for three surfaces. For the CF group, the respective figures were 660 protein peaks, with 381 significant at $P < 0.01$ and 315 at $P < 0.001$ for three surfaces (Table 2; for full summary, *see* Table E1 in the online supplement).

Although all four groups of patients yielded a substantial number of differential proteins compared with healthy control subjects, there were much closer similarities between the two obstructive airway diseases and between the two suppurative airway diseases. For asthma and COPD, only 16 proteins, on the three chip surfaces, were different at $P < 0.01$, and one protein was different at $P < 0.001$ (Table 2; Table E1). This latter protein, molecular weight (MW) 29 kD, was seen on the IMACNi surface (Figure 3A) but had low signal intensity. This marker has not yet been identified. CF and bronchiectasis demonstrated relatively similar biomarker profiles compared with each other, albeit the abundance of protein in the CF group was greater. Fifty-eight proteins differentiated the groups at $P < 0.01$ and 9 proteins differentiated the groups at $P < 0.001$ (Table 2; Table E1) over the three surfaces. Five of these nine proteins were observed on the IMACNi surface, two on CM10 and two on Q10. The marker that showed the greatest separation between the disease groups and had the highest signal intensity had an MW of 12.246 kD (Figure 3B). We have not identified this protein.

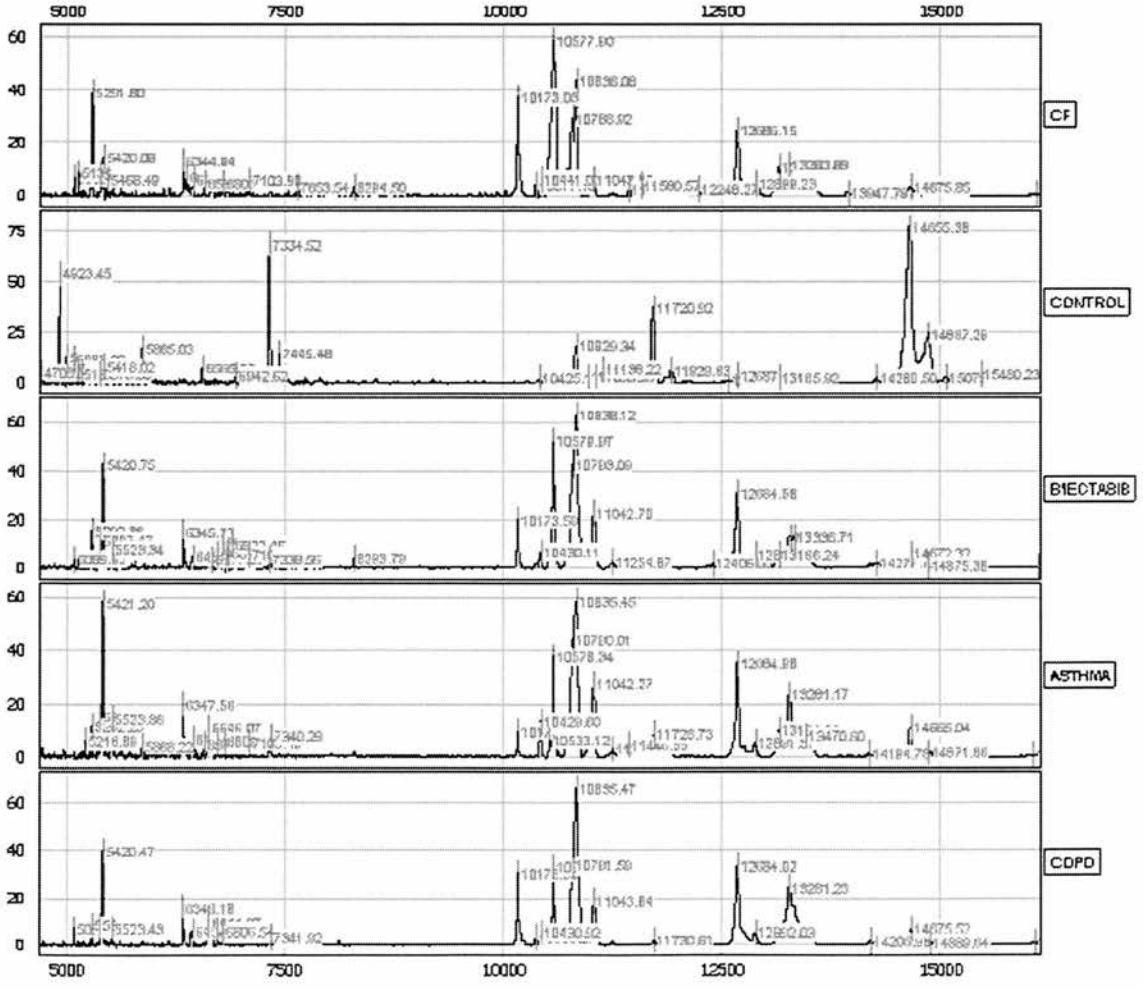


Figure 1. This figure demonstrates spectra from individual subjects on the immobilized metal affinity chromatography (IMAC) nickel surface in the mass range of 5 to 15 kD. Even in this mass range, there is an abundance of peptide peaks, and noticeable differences are observed between groups. B'iectasis = bronchiectasis; CF = cystic fibrosis; COPD = chronic obstructive pulmonary disease.

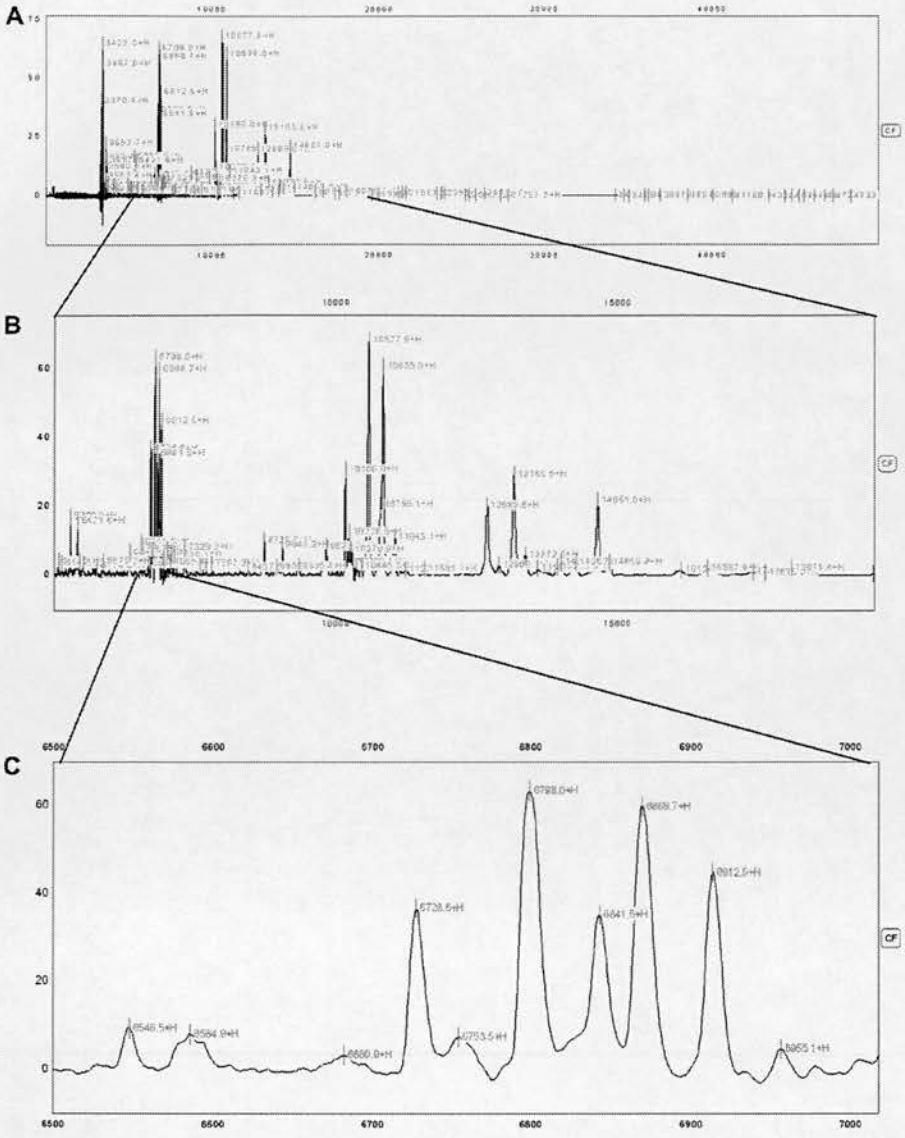


Figure 2. This figure demonstrates spectra from an individual with cystic fibrosis (CF) on the CM10 (cationic exchange) surface shown at different magnifications demonstrating the abundance of peaks in an individual sample. (*A*) demonstrates the entire spectrum (3,000–50,000 D); each label (most of which cannot be discerned individually at this magnification) represents a peak with signal-to-noise ratio of more than 3. (*B*) demonstrates some magnification to cover the spectrum from 5,000 to 20,000 D; again, most of the individual labels cannot be seen clearly at this magnification. (*C*) demonstrates magnified spectrum from 6,500 to 7,000 D, and at this level the individual peaks can be easily discerned.

The top 20 protein peaks that showed the most statistically significant differences between individual disease groups and healthy controls are shown in Table E2, indicating their molecular weight and the chip surface on which they were identified. There are common peaks found in asthma and COPD, and CF and bronchiectasis.

Biomarker Identification

The proteins we have formally identified from the induced sputum samples are calgranulin A, calgranulin B, calgranulin C, Clara cell secretory protein (CCSP, CC16, CCSP10, uteroglobin), lysosyme c, proline rich salivary peptide, cystatin s, and hemoglobin α (Table 3).

Calgranulins A, B, and C were highly abundant in CF and bronchiectasis, but were also seen in the other disease groups. These proteins are represented by mass spectral peaks at 10.831 kD, 12.700 kD, and 10.100 kD, respectively. These peaks were preferentially expressed on the IMACNi surface but were also bound to the weak cation exchange surface. Some binding of calgranulin A was also demonstrated on the anion exchange surface. A separate form of calgranulin A with a lower molecular weight (10.574 kD) was seen in the CF and bronchiectasis groups. This was identified by mass fingerprinting of both molecular weights after purification on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). The relative expression of calgranulin A across all groups is shown in Figure 4A and the relative expression of the smaller form of calgranulin A in Figure 4B. The presence of calgranulin A was confirmed with Western blotting in pooled sputum samples from all disease and control groups (Figure 4C).

In contrast to the calgranulins, CCSP was in lower abundance in all disease groups compared with controls, but to the greatest extent in CF and bronchiectasis. This had a mass spectral peak of 7.900 kD on the CM10 surface. The relative expression of CCSP across all groups is shown on Figure 5. CCSP was confirmed on Western blot (data not shown).

Correlation of Biomarkers with FEV_1 and Sputum Cytology

In the CF group, the most abundant biomarker, calgranulin A, showed a weak inverse correlation with FEV_1% predicted, which failed to reach statistical significance (Spearman r = 0.63, P = 0.08). There was no correlation of calgranulin A with the percentage of sputum neutrophils (data not shown).

TABLE 2. THE TOTAL NUMBER OF PEAKS DETECTED BY SELDI-TOF

	IMAC Nickel	Weak Cation Exchange (CM10)	Anion Exchange (Q10)
Asthma vs. control	4 (n = 22 vs. 20)	16 (n = 20 vs. 19)	36 (n = 16 vs. 18)
COPD vs. control	11 (n = 23 vs. 20)	13 (n = 22 vs. 19)	26 (n = 21 vs. 18)
CF vs. control	108 (n = 28 vs. 19)	111 (n = 22 vs. 19)	96 (n = 22 vs. 19)
Bronchiectasis vs. control	112 (n = 19 vs. 20)	103 (n = 17 vs. 19)	99 (n = 18 vs. 19)
CF vs. bronchiectasis	19 (n = 28 vs.19)	18 (n = 28 vs. 17)	21 (n = 22 vs. 19)
Asthma vs. COPD	10 (n = 23 vs. 22)	5 (n = 21 vs. 20)	1 (n = 21 vs. 25)

Definition of abbreviations: CF = cystic fibrosis; COPD = chronic obstructive pulmonary disease; SELDI-TOF = surface-enhanced laser desorption/ionization time-of-flight.

Significantly different ($P < 0.001$) for disease versus control are shown. The two suppurative airway diseases, CF and bronchiectasis, and the two inflammatory airway diseases, asthma and COPD, were also compared in terms of numbers of differentiating proteins ($P < 0.01$). Proteins could be increased or decreased in their abundance. Spectra that failed normalization were excluded from analysis (*see* text). n = number of normalized spectra used in each analysis, as per order in table legend. The spectra failing to normalize were not the same for each analysis. The data are displayed for each of the three chip surfaces that were used in the study.

Longitudinal Assessment of Identified Biomarker and Application of Specific ELISA

There were decreases in the levels of both calgranulin A and B, as assessed by SELDI-TOF, during treatment of an exacerbation of CF (Figures 6A and 6B; $P < 0.01$). Calprotectin (heterodimer of calgranulins A and B), measured by ELISA, similarly showed significant reduction (Figure 6C).

DISCUSSION

The use of SELDI-TOF MS has allowed us to generate sputum protein profiles of several diseases. We have shown there are substantial numbers of potential protein biomarkers that differentiate patients with inflammatory airway diseases from healthy subjects. We have proceeded to identify several of these candidate biomarkers and, using the example of calgranulins A and B, have demonstrated clinical relevance with longitudinal evaluation during altered disease activity. We have demonstrated that, having identified a biomarker by primary screening rather than empirical preselection, a standard assay, ELISA, can then be applied to the same clinical samples and deliver quantitative measures of the biomarker. Thus, screening with high-technology SELDI-TOF proteomics can be converted into clinically applicable measures.

Because this is the first study of its kind to investigate the proteomics of induced sputum using SELDI-TOF, we sought to determine the reproducibility of this assay. The average intraassay CV was 22.41% and average interassay CV was 16.25%. This compares well to published data for serum (intraassay CV of 15.6%, interassay variation of 24.4% [19], urine (intraassay CV, 8–30%) (32), and saliva (intraassay CV, 18%, and interassay CV, 31%) (33).

Previous studies have demonstrated the utility of SELDI-TOF in the diagnosis of illnesses in a range of body fluids. We used the fluid phase of sputum as a noninvasive means of assessing the airways. Although previous work has suggested a correlation of induced sputum with bronchial washings but not BAL (34), and therefore suggests that it reflects the pathophysiology of more central airways, induced sputum has been demonstrated useful in inflammatory respiratory diseases (11, 12, 35–46). Therefore, we would conclude that the proteomic assessment of induced sputum is a valid means of assessing airway disease.

As described previously, SELDI-TOF technology has been used as a technique in a number of inflammatory and neoplastic diseases, mainly using serum as a sampling medium. It was suggested as a diagnostic platform using bioinformatic algorithms to separate subjects with ovarian cancer from healthy control subjects (15). This initial approach, however, was widely criticized in the literature because the results were not reproducible when the same data sets were examined by independent investigators (21). Baggerly and colleagues (21) drew particular notice to the impact of different modes of data preparation after acquisition, such as baseline subtraction in some groups of data and not in others. They also suggested that inaccuracies in sample collection protocol and mass calibration could lead to misinformed results in these sorts of experiments. To ensure uniformity in this study, all data in our analysis underwent the same steps of preparation before analysis. We also ensured that all sample preparation was uniform as outlined in METHODS and *below*. Mass calibration was performed before each experiment using protein and peptide standards (Ciphergen All in One protein and peptide standards; Ciphergen). Furthermore, the widely criticized Petricoin study also used mass spectral data at less than 1,000 D, a mass range at

TABLE 3. PROTEIN IDENTIFICATIONS CONFIRMED FROM PRESENT STUDY

Protein	Molecular Weight (D)	Accession Number	Corresponding SELDI Peak (D)	Confirmed by PMF	Confirmed by MS/MS	Confirmed By Antibody	Direction of Change in Suppuration
Calgranulin A	10,834	P05109	10,834, 10,596	Yes	Yes	Yes	Increased
Calgranulin B	12,960	P06702	12,960, 13,200	Yes	Yes	Yes	Increased
Calgranulin C	10,100	P80511	10,100	Yes	Yes	No	Increased
Clara cell secretory protein	7,900	P11684	7,900	Yes	Yes	Yes	Decreased
Proline rich salivary peptide	8,188	P02814	8,119	No	Yes	No	Decreased
Lysosyme C precursor	16,537	P61626	14,600	Yes	Yes	No	No Change
Cystatin s	16,204	P01036	16,079	No	Yes	No	No Change
hemoglobin alpha	15,117	P69905	15,080	No	Yes	No	No Change

Definition of abbreviations: PMF = peptide mass fingerprinting; SELDI = surface-enhanced laser desorption/ionization.

Proteins were identified by trypsin digest and PMF and well as tandem MS/MS. When available, antibodies were used to confirm protein identification by Western blot. Molecular weight refers to the theoretical molecular weight of each protein as derived from sequence. Corresponding SELDI peak refers to the protein peak seen on SELDI analysis of sputum fluid phase (differences in molecular weight may represent post-translational modifications).

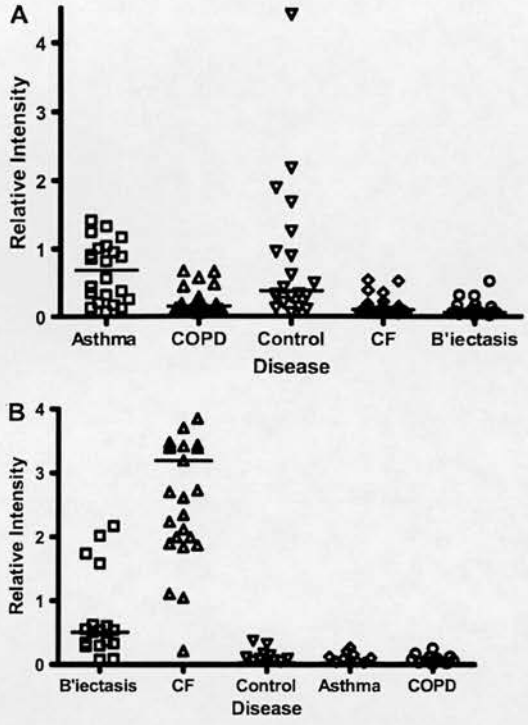


Figure 3. (*A*) The protein that gave the statistically greatest differentiation between asthma and chronic obstructive pulmonary disease (COPD) ($P < 0.001$) had a MW of 29 kD, and was detected on the IMAC nickel (IMACNi) surface. The individual data and the *median bars* are shown. The data are for 22 patients with asthma and 23 patients with COPD (*see* explanation in footnote to Table 2). Other disease groups are demonstrated for comparison. (*B*) The protein that gave the statistically greatest differentiation between cystic fibrosis (CF) and bronchiectasis (B'iectasis) ($P < 0.001$) had an MW of 12.246 kD, and was detected on the IMACNi surface. The individual data and the *median bars* are shown. The data are for 28 patients with CF and 19 patients with bronchiectasis (*see* explanation in footnote to Table 2). Other disease groups are demonstrated for comparison.

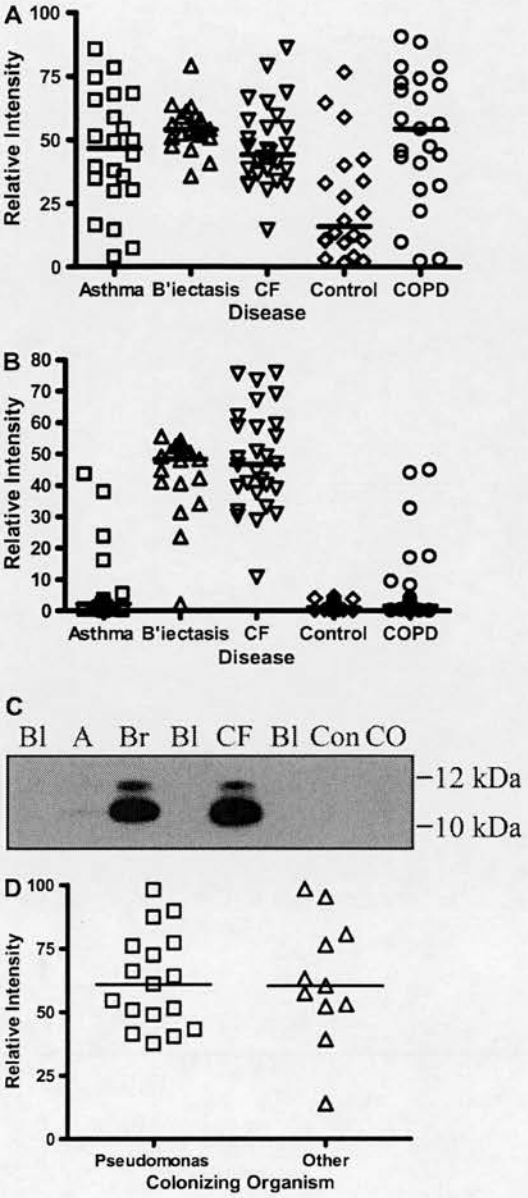


Figure 4. Calgranulin A in all disease groups: (*A*) shows the relative expression, on surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF), of the 10,831-D protein, which was elevated ($P < 0.001$) in all disease groups versus control; (*B*) shows the expression of the 10,576-D form of calgranulin A, which was specifically elevated in patients with cystic fibrosis (CF) and bronchiectasis (B'iectasis) ($P < 0.001$ vs. all other groups) (the data in *A* and *B* were obtained using the IMAC Nickel surface); (*C*) is a Western blot of calgranulin A. The blot represents the overall abundance of calgranulin A from both 10,834- and 10,576-D forms in the sample groups (*lanes* are labeled as Bl = blank, A = asthma; Br = bronchiectasis; CF = cystic fibrosis; Con = control; CO = COPD). Note the strong staining for CF and bronchiectasis groups and very weak staining in asthma. (*D*) demonstrates the effect of bacterial colonization on the signal intensity of the 10,576-D form of calgranulin A; the majority of patients were colonized with *Pseudomonas aeruginosa* (shown in the first column labeled "Pseudomonas"); other organisms included *Staphylococcus aureus*, *Haemophilus*, *Stenotrophomonas*, and *Burkholderia* (labeled "Other").

which the discrimination of genuine protein peaks from background noise may be difficult. Our approach of excluding data from below 4,000 D from analysis further strengthens the findings of this study. Other studies using SELDI-TOF as a diagnostic platform in infectious diseases using more robust sample collection and data preparation algorithms have proved more promising (19, 20). However, in this study, we used SELDI-TOF as a high-throughput screening platform for biomarker discovery rather than as a primary diagnostic assay. Although, on initial observation, our data demonstrate a large number of differentiating peaks when we look at the combined data set, the actual clinical significance of this observation is less clear and should be interpreted with caution.

Previous work by our group suggested an optimum mass range for respiratory biomarker discovery with this platform to be 3 to 20 kD (25). This may lead to biomarkers of a higher mass being ignored. Other proteomics methods, such as two-dimensional gel electrophoresis and immunocapture, may be better in assessing larger molecular weight proteins, and has been applied to CF (27, 29).

The largest numbers of differentiating proteins between disease and control were found in CF and bronchiectasis. This may suggest greater numbers of inflammatory and disease-

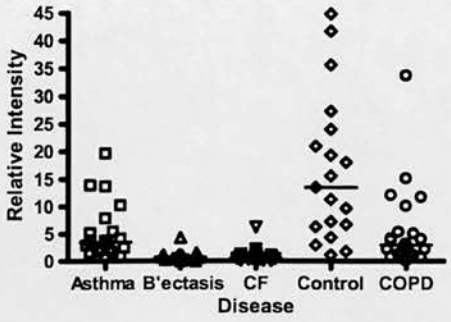


Figure 5. Clara cell secretory protein, molecular weight (MW) 7.9 kD, was in lower abundance for all disease groups compared with controls, but to the greatest extent in cystic fibrosis (CF) and bronchiectasis (B'ectasis). These data were obtained using the cation exchange (CM10) surface. The individual data and the *median bars* are shown. The data are for 20 patients with asthma, 17 with bronchiectasis, 22 with CF, 22 patients with chronic obstructive pulmonary disease (COPD), and 19 control subjects (*see* explanation in footnote to Table 2).

related proteins in these groups or, alternatively, samples obtained from these groups had greater abundance of protein overall. Sputum samples obtained from subjects with CF and bronchiectatic subjects were of greater volume and better quality subjectively. However, selected mucus plugs were used for analysis and processed with equal ratios of buffer as previously described (46). Moreover, to ensure samples were comparable, supernatants were adjusted to a total protein concentration of 1 mg/ml before applying to chip surfaces. Also, mass spectral data were normalized to total ion current before data analysis, thus minimizing effects of possible variation in protein binding to chip surfaces. After normalization, samples were excluded because they had absent or very poor signals. We would therefore suggest that the differences expressed between groups represent real differences in protein expression rather than confounding due to issues of sample quality and abundance.

Our data revealed large numbers of potential biomarkers. The preset parameters we created may have limited the possible number. A lower limit cutoff of 4 kD may have been too harsh. Indeed, our spectra showed the human α defensins in all groups, but these were excluded from analysis because their average mass was less than 4 kD. Alternatively, we may have overestimated true numbers of differentiating markers because some proteins were detected on more than one chip surface. Also, a proportion of some proteins may be doubly protonated. Because the "time of flight" is related to both MW and charge, such proteins will appear "twice," once with half the MW of the original protein. Furthermore, different peaks may represent cleavage products of the same (higher molecular weight) proteins and this may in part explain the high numbers of potential differentiating peaks we recorded, particularly when comparing our CF and bronchiectasis groups with control subjects.

Despite finding a large number of peaks differentiating CF and bronchiectasis from controls, we were surprised not to have identified any of these as known biomarkers of inflammation, such as IL-8 or neutrophil elastase. In our study, SELDI-TOF was most efficient at demonstrating peaks in the 5–20-kD range and this may in part explain these findings as the predicted molecular weights of neutrophil elastase is 29.5 kD (47). Furthermore, the chip surface chemistry and binding conditions may simply not have favored preferential selection of IL-8.

Despite the limitations in this technique, we found large numbers of potential biomarkers from which we have so far identified a modest number, although further identification of

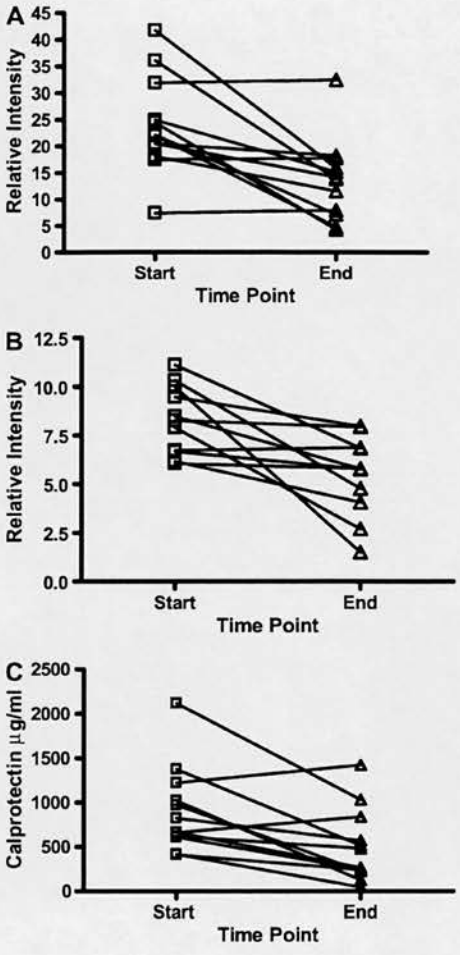


Figure 6. The results for calgranulins A and B/calprotectin for 12 patients with cystic fibrosis are shown at the onset of an infective exacerbation and at the completion of antibiotic therapy, when there was a decrease ($P < 0.01$) of the proteins measured. (*A*) shows data for calgranulin A; (*B*) shows data for calgranulin B; (*C*) shows data for calprotectin measured by ELISA.

other peaks is ongoing. We used one of these biomarkers, calprotectin (calgranulin A and B), to show that it was possible to monitor disease activity and to apply an ELISA to the same clinical samples, obtaining the same results as with MS.

In all disease groups, we identified calgranulin A at higher levels than in control subjects. This S100 protein is produced by neutrophils, macrophages, and epithelial cells. In the CF and bronchiectasis groups, a protein at 10.574 kD was also identified as calgranulin A. Theoretical removal of glutamic acid and lysine from the N terminus of calgranulin A would result in an identical mass shift from 10.831 to 10.574 kD. Calgranulin 10.574 may therefore be the product of specific cleavage or post-translational modification in CF and bronchiectasis. Whether this finding may be of functional significance is as yet unclear. Calgranulins A and B have also been described recently in BAL fluid from subjects with CF (25, 26); a similar finding of a separate peak representing calgranulin A at a lower molecular weight was also described in BAL fluid by McMorran and colleagues (26).

Calgranulin has previously been described in the sera of subjects who were homo- and heterozygous for CF mutations and was referred to as CF antigen (48). Calgranulin A may

provide a robust and sensitive marker of airway inflammation in CF. The likely source of calgranulin A is from neutrophils in the airway, although in this study we failed to demonstrate any significant correlation between neutrophil % in sputum and calgranulin A as measured by SELDI-TOF. This may in part be explained by the finding that the majority of the patients with CF had a profound neutrophilia in sputum in excess of 95%, regardless of other clinical details. Furthermore, there was a weak relationship between decreasing FEV_1 and higher levels of calgranulin A, implying that calgranulin A may be a marker of disease severity in CF. We replicated our SELDI-TOF results using an ELISA to calprotectin, the heterodimer of calgranulins A and B. Thus, a more practical assay can be applied to induced sputum and yield the same clinical information. Calprotectin has previously been recognized as a marker in other inflammatory disorders, including inflammatory bowel disease and arthritis (49–51). Calgranulin C, another S100 protein, was also identified in our samples. This is in keeping with previous work demonstrating its presence in sputum and its potential usefulness as a biomarker in serum for CF (52).

A second abundant protein identified showed opposite effects to calgranulin A. CCSP was reduced in all disease groups compared with controls. CCSP is an antiinflammatory protein mainly expressed in the epithelial cells of the airways. Low serum levels have been reported in patients with asthma (53), and low nasal lavage levels in patients with allergic rhinitis (54). CCSP has a number of possible activities, including inhibition of phospholipase A2, chelation of calcium, and down-regulation of IFN-γ, IL-1, and tumor necrosis factor-α. Our findings are consistent with these previous studies, and raise the possibility that resolution of inflammatory processes might be monitored by rising levels of CCSP.

In conclusion, we have demonstrated the utility of SELDI-TOF MS as a tool for biomarker discovery in induced sputum. We have positively identified proteins of biological significance in the fluid phase of sputum. These proteins may form the basis of clinical point assays to assess the presence and activity of inflammation in a variety of lung diseases, with a particular emphasis being placed on disease monitoring.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors gratefully acknowledge the support of the Cystic Fibrosis Trust in their sponsorship of this study through the U.K. CF Gene Therapy Consortium. They thank Nathan Harris for his technical advice and support in the use of SELDI-TOF.

References

1. Franklin PJ, Taplin R, Stick SM. A community study of exhaled nitric oxide in healthy children. *Am J Respir Crit Care Med* 1999;159:69–73.
2. Ho LP, Innes JA, Greening AP. Exhaled nitric oxide is not elevated in the inflammatory airways diseases of cystic fibrosis and bronchiectasis. *Eur Respir J* 1998;12:1290–1294.
3. Gaston B, Drazen JM, Loscalzo J, Stamler JS. The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 1994;149:538–551.
4. Ho LP, Wood FT, Robson A, Innes JA, Greening AP. Atopy influences exhaled nitric oxide levels in adult asthmatics. *Chest* 2000;118:1327–1331.
5. Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Baraldi E, Becher G, van Beurden WJ, Corradi M, Dekhuijzen R, *et al.* Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J* 2005;26:523–548.
6. Hunt JF, Fang K, Malik R, Snyder A, Malhotra N, Platts-Mills TA, Gaston B. Endogenous airway acidification: implications for asthma pathophysiology. *Am J Respir Crit Care Med* 2000;161:694–699.
7. Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 1994;343:133–135.
8. Rutgers SR, van der Mark TW, Coers W, Moshage H, Timens W, Kauffman HF, Koeter GH, Postma DS. Markers of nitric oxide metabolism in sputum and exhaled air are not increased in chronic obstructive pulmonary disease. *Thorax* 1999;54:576–580.
9. Smith AD, Cowan JO, Brassett KP, Herbison GP, Taylor DR. Use of exhaled nitric oxide measurements to guide treatment in chronic asthma. *N Engl J Med* 2005;352:2163–2173.
10. Tate S, MacGregor G, Davis M, Innes JA, Greening AP. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax* 2002;57:926–929.
11. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, Wardlaw AJ, Pavord ID. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;360:1715–1721.
12. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. *J Pediatr* 2002;141:811–817.
13. Zhang L, Yu W, He T, Yu J, Caffrey RE, Dalmasso EA, Fu S, Pham T, Mei J, Ho JJ, *et al.* Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* 2002;298: 995–1000.
14. Xiao Z, Adam BL, Cazares LH, Clements MA, Davis JW, Schellhammer PF, Dalmasso EA, Wright GL Jr. Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. *Cancer Res* 2001; 61:6029–6033.
15. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, *et al.* Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 2002; 359:572–577.
16. Kozak KR, Su F, Whitelegge JP, Faull K, Reddy S, Farias-Eisner R. Characterization of serum biomarkers for detection of early stage ovarian cancer. *Proteomics* 2005;5:4589–4596.
17. Ranganathan S, Williams E, Ganchev P, Gopalakrishnan V, Lacomis D, Urbinelli L, Newhall K, Cudkowicz ME, Brown RH Jr, Bowser R. Proteomic profiling of cerebrospinal fluid identifies biomarkers for amyotrophic lateral sclerosis. *J Neurochem* 2005;95:1461–1471.
18. Peronnet E, Becquart L, Poirier F, Cubizolles M, Choquet-Kastylevsky G, Jolivet-Reynaud C. SELDI-TOF MS analysis of the cardiac troponin I forms present in plasma from patients with myocardial infarction. *Proteomics* 2006;6:6288–6299.
19. Agranoff D, Fernandez-Reyes D, Papadopoulos MC, Rojas SA, Herbster M, Loosemore A, Tarelli E, Sheldon J, Schwenk A, Pollok R, *et al.* Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. *Lancet* 2006;368:1012–1021.
20. Papadopoulos MC, Abel PM, Agranoff D, Stich A, Tarelli E, Bell BA, Planche T, Loosemore A, Saadoun S, Wilkins P, *et al.* A novel and accurate diagnostic test for human African trypanosomiasis. *Lancet* 2004;363:1358–1363.
21. Baggerly KA, Morris JS, Coombes KR. Reproducibility of SELDI-TOF protein patterns in serum: comparing datasets from different experiments. *Bioinformatics* 2004;20:777–785.
22. White MY, Gundry RL, Cordwell SJ. When does a fingerprint constitute a diagnostic? *Lancet* 2006;368:971–973.
23. Merkel D, Rist W, Seither P, Weith A, Lenter MC. Proteomic study of human bronchoalveolar lavage fluids from smokers with chronic obstructive pulmonary disease by combining surface-enhanced laser desorption/ionization-mass spectrometry profiling with mass spectrometric protein identification. *Proteomics* 2005;5:2972–2980.
24. Kriegova E, Melle C, Kolek V, Hutyrova B, Mrazek F, Bleul A, du Bois RM, von Eggeling F, Petrek M. Protein profiles of bronchoalveolar lavage fluid from patients with pulmonary sarcoidosis. *Am J Respir Crit Care Med* 2006;173:1145–1154.
25. Macgregor G, Gray RD, Hilliard TN, Imrie M, Boyd AC, Alton EW, Bush A, Davies JC, Innes JA, Porteous DJ, *et al.* Biomarkers for cystic fibrosis lung disease: application of SELDI-TOF mass spectrometry to BAL fluid [in press]. *J Cyst Fibros* 2008.
26. McMorran BJ, Patat SA, Carlin JB, Grimwood K, Jones A, Armstrong DS, Galati JC, Cooper PJ, Byrnes CA, Francis PW, *et al.* Novel neutrophil-derived proteins in bronchoalveolar lavage fluid indicate an exaggerated inflammatory response in pediatric cystic fibrosis patients. *Clin Chem* 2007;53:1782–1791.
27. Sloane AJ, Lindner RA, Prasad SS, Sebastian LT, Pedersen SK, Robinson M, Bye PT, Nielson DW, Harry JL. Proteomic analysis of sputum from adults and children with cystic fibrosis and from control subjects. *Am J Respir Crit Care Med* 2005;172:1416–1426.
28. Nicholas B, Skipp P, Mould R, Rennard S, Davies DE, O'Connor CD, Djukanovi R. Shotgun proteomic analysis of human-induced sputum. *Proteomics* 2006;6:4390–4401.

29. Pedersen SK, Sloane AJ, Prasad SS, Sebastian LT, Lindner RA, Hsu M, Robinson M, Bye PT, Weinberger RP, Harry JL. An immunoproteomic approach for identification of clinical biomarkers for monitoring disease: application to cystic fibrosis. *Mol Cell Proteomics* 2005; 4:1052–1060.
30. Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, Gleich GJ, Dolovich J, Hargreave FE. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308–317.
31. Efthimiadis A, Spanevello A, Hamid Q, Kelly MM, Linden M, Louis R, Pizzichini MMM, Pizzichini E, Ronchi C, Van Overveld F, *et al.* Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. *Eur Respir J* 2002;20(37 Suppl):19S–23S.
32. Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P. Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Kidney Int* 2004;65:323–332.
33. Schipper R, Loof A, de Groot J, Harthoorn L, Dransfield E, van Heerde W. SELDI-TOF-MS of saliva: methodology and pre-treatment effects. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007;847:45–53.
34. Moodley YP, Krishnan V, Lalloo UG. Neutrophils in induced sputum arise from central airways. *Eur Respir J* 2000;15:36–40.
35. Berry MA, Parker D, Neale N, Woodman L, Morgan A, Monk P, Bradding P, Wardlaw AJ, Pavord ID, Brightling CE. Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis. *J Allergy Clin Immunol* 2004;114:1106–1109.
36. Birring SS, Berry M, Brightling CE, Pavord ID. Eosinophilic bronchitis: clinical features, management and pathogenesis. *Am J Respir Med* 2003;2:169–173.
37. Birring SS, Parker D, McKenna S, Hargadon B, Brightling CE, Pavord ID, Bradding P. Sputum eosinophilia in idiopathic pulmonary fibrosis. *Inflamm Res* 2005;54:51–56.
38. Brightling CE. Clinical applications of induced sputum. *Chest* 2006;129: 1344–1348.
39. Brightling CE, McKenna S, Hargadon B, Birring S, Green R, Siva R, Berry M, Parker D, Monteiro W, Pavord ID, *et al.* Sputum eosinophilia and the short term response to inhaled mometasone in chronic obstructive pulmonary disease. *Thorax* 2005;60:193–198.
40. Brightling CE, Monteiro W, Ward R, Parker D, Morgan MD, Wardlaw AJ, Pavord ID. Sputum eosinophilia and short-term response to prednisolone in chronic obstructive pulmonary disease: a randomised controlled trial. *Lancet* 2000;356:1480–1485.
41. Chalmers GW, Macleod KJ, Sriram S, Thomson LJ, McSharry C, Stack BH, Thomson NC. Sputum endothelin-1 is increased in cystic fibrosis and chronic obstructive pulmonary disease. *Eur Respir J* 1999;13:1288–1292.
42. Demedts IK, Morel-Montero A, Lebecque S, Pacheco Y, Cataldo D, Joos GF, Pauwels RA, Brusselle GG. Elevated MMP-12 protein levels in induced sputum from COPD patients. *Thorax* 2006;61:196–201.
43. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002;57:875–879.
44. Hargreave FE, Leigh R. Induced sputum, eosinophilic bronchitis, and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;160:S53–S57.
45. Pavord ID, Sterk PJ, Hargreave FE, Kips JC, Inman MD, Louis R, Pizzichini MM, Bel EH, Pin I, Grootendorst DC, *et al.* Clinical applications of assessment of airway inflammation using induced sputum. *Eur Respir J Suppl* 2002;37:40s–43s.
46. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, Dolovich J. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax* 1992;47:25–29.
47. Sinha S, Watorek W, Karr S, Giles J, Bode W, Travis J. Primary structure of human neutrophil elastase. *Proc Natl Acad Sci USA* 1987; 84:2228–2232.
48. van Heyningen V, Dorin J. Possible role for two calcium-binding proteins of the S-100 family, co-expressed in granulocytes and certain epithelia. *Adv Exp Med Biol* 1990;269:139–143.
49. Colombo C, Costantini D, Rocchi A, Cariani L, Garlaschi ML, Tirelli S, Calori G, Copreni E, Conese M. Cytokine levels in sputum of cystic fibrosis patients before and after antibiotic therapy. *Pediatr Pulmonol* 2005;40:15–21.
50. Schulze zur Wiesch A, Foell D, Frosch M, Vogl T, Sorg C, Roth J. Myeloid related proteins MRP8/MRP14 may predict disease flares in juvenile idiopathic arthritis. *Clin Exp Rheumatol* 2004;22:368–373.
51. Silberer H, Kuppers B, Mickisch O, Baniewicz W, Drescher M, Traber L, Kempf A, Schmidt-Gayk H. Fecal leukocyte proteins in inflammatory bowel disease and irritable bowel syndrome. *Clin Lab (Zaragoza)* 2005;51:117–126.
52. Foell D, Seeliger S, Vogl T, Koch HG, Maschek H, Harms E, Sorg C, Roth J. Expression of S100A12 (EN-RAGE) in cystic fibrosis. *Thorax* 2003;58:613–617.
53. Shijubo N, Itoh Y, Yamaguchi T, Sugaya F, Hirasawa M, Yamada T, Kawai T, Abe S. Serum levels of Clara cell 10-kDa protein are decreased in patients with asthma. *Lung* 1999;177:45–52.
54. Johansson S, Keen C, Stahl A, Wennergren G, Benson M. Low levels of CC16 in nasal fluid of children with birch pollen-induced rhinitis. *Allergy* 2005;60:638–642.

CHEST

Original Research

BIOMARKERS

Sputum Trace Metals Are Biomarkers of Inflammatory and Suppurative Lung Disease

Robert D. Gray, MB ChB; Andrew Duncan, PhD; Donald Noble, MD; Margaret Imrie, BSc; Dennis St. J. O'Reilly, MD; J. Alastair Innes, PhD; David J. Porteous, PhD; Andrew P. Greening, MD; and A. Christopher Boyd, PhD

Background: **Induced sputum cytology and protein biomarkers can be used to assess airways inflammation. Increases in sputum iron have been described in inflammatory lung disease. We hypothesized that other sputum metals may be affected by airways inflammation and investigated their potential value as biomarkers.**

Methods: **Sputum was obtained from 20 healthy control subjects and from patients with inflammatory pulmonary diseases (23 with cystic fibrosis [CF], 16 with bronchiectasis, 17 with asthma, and 23 with COPD), and iron, zinc, manganese, and copper were measured. Fourteen patients with CF were also studied through an exacerbation cycle.**

Results: **Sputum zinc and iron were elevated in CF and non-CF bronchiectasis vs controls ($P < .001$, zinc; $P < .01$ iron). Manganese was elevated in asthma ($P < .01$) and bronchiectasis ($P < .05$) vs controls. Copper was elevated in CF vs controls ($P < .05$). Zinc decreased ($P < .01$) following treatment of CF exacerbation. In subjects with CF zinc levels correlated with other biomarkers.**

Conclusions: **These results suggest a relationship of high concentrations of total zinc and iron with airways inflammation in CF and non-CF bronchiectasis, with longitudinal changes being observed in CF. Further work is required to elucidate potential inflammatory mechanisms related to these observations.** ***CHEST 2010; 137(3):635–641***

Abbreviations: CF = cystic fibrosis; IL = interleukin; MPO = myeloperoxidase; PBS = phosphate-buffered saline; SOD = superoxide dismutase

The presence of a proinflammatory milieu in the airway is important for the development of a range of inflammatory lung diseases. Adequate assessment of inflammatory cells, cytokines, chemokines, and antiinflammatory molecules is essential for understanding, monitoring, and treating these disorders. Induced sputum provides a noninvasive means of investigating airways inflammation.[1] Induced sputum has a fluid phase containing a heterogeneous mix of proteins and nonorganic substances. A number of cytokines, including interleukin (IL)-8, have been measured,[2] albeit with significant interstudy variation in reported levels.[3] The nonorganic component, however, has been largely neglected.

Previous work performed by this group has demonstrated high levels of the neutrophil protein calprotectin in the sputum[4] and lavage fluid[5] of patients with CF and non-CF bronchiectasis. Calprotectin possesses antimicrobial properties mediated by the chelation of zinc.[6,7] It has been demonstrated recently that calprotectin is recruited to staphylococcal abscesses in a neutrophil-dependent manner and inhibits *Staphylococcus*

Manuscript received May 1, 2009; revision accepted August 20, 2009.

Affiliations: From the School of Molecular and Clinical Medicine (Drs Gray, Noble, Innes, Porteous, Greening, and Boyd and Ms Imrie), University of Edinburgh, Western General Hospital, Edinburgh; and the Scottish Trace Element and Micronutrient Reference Laboratory, Department of Clinical Biochemistry (Drs Duncan and O'Reilly), Glasgow Royal Infirmary, Scotland.

Funding/Support: This study was funded by the Cystic Fibrosis Trust (R.D.G. and A.C.B.). R.D.G. was supported in part by the Medical Research Council (Programme Grant G9313618). D.N. was supported by an unrestricted grant from GlaxoSmithKline.

Correspondence: R. D. Gray, MB, ChB, Medical Genetics, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Edinburgh, Scotland; e-mail: r.d.gray@ed.ac.uk

DOI: 10.1378/chest.09-1047

aureus growth through the chelation of zinc and manganese.[8]

Increased levels of total iron and iron-binding proteins have been reported in the sputum of patients with cystic fibrosis (CF) and COPD.[9-12] Scavenging of free iron is an important component of antimicrobial defense mechanisms against organisms such as *Pseudomonas aeruginosa*,[13] an important pathogen in chronic lung disease. Although iron is clearly an important inorganic substance in the airway, other metals, such as zinc, may also be important; for example, increased zinc levels alter the sensitivity of *Pseudomonas*, an organism particularly relevant to CF lung disease, to antibiotics.[14] Therefore the measurement of trace elements in sputum may allow mechanistic insight into diseases such as CF. Furthermore, trace elements, likely to be less susceptible to protease activity, may allow more reliable measurement of inflammation in the airway. Thus we feel that measurement of trace elements may allow a more robust sputum measurement of airways disease as well as giving further insight into lung inflammation in diseases such as CF.

Based on our previous findings of increased levels of calprotectin, a known chelator of zinc, in the airway and previous observations related to iron in the CF airway we hypothesized that trace metal concentrations, in particular zinc and iron, would be elevated in CF and non-CF bronchiectasis compared with control populations. We also hypothesized that levels of trace element would change following disease intervention in CF. Total concentrations of zinc, iron, copper, and manganese were measured in subjects with CF, bronchiectasis, COPD, and asthma, and in normal controls. Furthermore, concentrations of trace elements were compared with previously described markers of inflammation in CF, including calprotectin.

Materials and Methods

Subjects

Induced sputum was obtained from patients attending the Respiratory Unit at the Western General Hospital, Edinburgh. Approval was obtained from the local regional ethics committee. Twenty-three patients with CF, 16 with non-CF bronchiectasis, 17 with asthma, 23 with COPD, and 20 healthy controls were studied. In order to monitor the effects of a changing state of inflammation, sputum samples were obtained from 14 additional patients with CF during CF exacerbation. Exacerbation samples were taken within 24 hours of starting antibiotic therapy and at the end of treatment.

Reagents Used

All reagents were purchased from Sigma (Sigma; Gillingham, UK) unless otherwise stated. Ultrapure, high-performance liquid chromatography-grade water was used for all experiments and preparations. Aristar nitric acid was used in the trace element assay.

Sputum Induction

Sputum induction was performed by a standard method as previously described.[15-17] In brief, patients inhaled nebulized hypertonic saline at concentrations of 3%, 4%, and 5% and were asked to expectorate after each inhalation until a sample was obtained. Patients with CF underwent induction unless freely expectorating in keeping with previous CF sputum studies.[18,19] Nine of 23 subjects with CF spontaneously produced sputum without induction.

Sputum Processing

Sputum was processed within 2 hours of collection as described previously.[15] In brief, sputum plugs were harvested and processed with 4 × weight/volume of 0.1% dithiothreitol in phosphate-buffered saline (PBS), after which 4 × weight/volume of PBS was added. Samples were filtered through 48-μm mesh and centrifuged at 1,200 rpm to remove the cells. Supernatant was stored at −80°C until further analysis without protease inhibitor. The cell pellet was resuspended in PBS and used for cytospin preparation. Cytospins were stained with May-Grunwald-Giemsa for differential cell counting. All cell counts were expressed as percentage of the population counted. Total cell count was not performed prior to cytospin. All samples used in this study contained < 40% squamous cells.

Trace Element Assay

Trace element assay was performed in the Scottish National Trace Element Reference Laboratory, Glasgow, Scotland. A four-point calibration was used (0, 100, 500, 1,000 μg/L copper, iron, zinc, manganese in 1% nitric acid). Sputum samples were centrifuged at 800 g for 5 min and 200 μL of sample was then diluted with 2 mL internal standard solution (100 μ/L yttrium in 1% nitric acid) and mixed by inversion. Internal accuracy was assessed by use of two aqueous certified reference materials, TMDA62 and TMDA64 (Prochem; Chessington, England). Trace element levels were measured by inductively coupled plasma optical emission spectrometry using a VISTA AX (Varian; Oxford, England).

Measurement of Sputum IL-8, Myeloperoxidase, and Calprotectin

To compare the results of metals in sputum with biochemical markers of inflammation, comparisons with IL-8, myeloperoxidase (MPO), and calprotectin were made in the CF group. Immunoassays used commercially available sandwich enzyme-linked immunosorbent assay kits, following the manufacturer's instructions. The kits used were IL-8 (Biosource Europe SA; Nivelles, Belgium), MPO (Assay Designs Inc; Ann Arbor, MI), and Calprotectin (Buhlmann Laboratories, AG; Schonenbuch, Switzerland).

Data Analysis

Data analysis was carried out on GraphPad Prism software (GraphPad; La Jolla, CA) for Windows. Cross-sectional data were nonnormally distributed and were analyzed by Kruskal-Wallis analysis of variance and Dunn multiple comparison test. $P < .05$ was considered significant. For longitudinal analysis of zinc and iron data a paired Student t test was performed. For correlation, data were subjected to Spearman rank analysis.

Results

Subject Demographics

The demographic profiles and sputum cytology of each group are shown in Table 1. The CF group was

Table 1—*Demographics and Sputum Cytology for Subjects in Cross-Sectional and Longitudinal Studies*

Disease	No.	Male, No.	Age, y	FEV_1 % Predicted	Neutrophil, %	Eosinophil, %
Controls	20	6	36.9 (2.5)	105.8 (2.6)	55 (4.3)	2.9 (0.5)
Asthma	17	5	51.9 (3.3)	80 (5.6)	60 (6.1)	16.2 (5.2)
COPD	23	16	66.0 (1.4)	55.0 (4.0)	81 (2.6)	4.4 (1.6)
CF	23	14	26.3 (2.0)	59.1 (4.4)	92 (2.0)	2.4 (0.5)
Bronchiectasis	16	1	62.3 (2.1)	70.4 (8.5)	84 (4.0)	2.1 (1.3)
CF exacerbation	14	8	25.6 (2.4)	41.0 (4.4)	98.3 (0.6)	n/d
CF recovery	14	8	25.6 (2.4)	46.3 (4.5)	96.4 (0.9)	n/d

Data are displayed as mean (SEM). CF = cystic fibrosis; n/d = not done.

younger than the control and other disease groups ($P < .01$). Of the COPD group 10 were current smokers, nine ex-smokers, and four gave no information on current smoking status. Of the patients with CF, 14 were colonized with *P aeruginosa*, the other patients being colonized by a variety of organisms, including *Stenotrophomonas maltophilia* and *Burkholderia cenocepacia* species.

Assay Reproducibility

Pooled samples of five subjects in each group of control, CF, and non-CF bronchiectasis were assayed in two separate runs for zinc levels with coefficients of variance of 14.7%, 2.7%, and 5.4%, respectively, giving an average coefficient of variation of 7.6%.

Sputum Trace Element Levels in Cross-Sectional Data

The absolute concentrations of zinc, iron, and manganese are displayed in Table 2 in μg/L. Sputum zinc concentration was at least fourfold higher in CF and non-CF bronchiectasis than controls ($P < .001$). Concentrations in CF and non-CF bronchiectasis were also higher than in asthma and COPD ($P < .05$).

Sputum iron was at least twofold higher in CF and non-CF bronchiectasis than controls ($P < .01$) and COPD ($P < .05$). Levels were higher in the COPD and asthma groups vs controls but did not reach statistical significance. Current smoking status had no significant effect on the zinc or iron levels in the COPD group (Fig 1).

Sputum manganese differentiated non-CF bronchiectasis but not CF from control ($P < .05$). Sputum manganese also differentiated subjects with asthma from control subjects ($P < .01$). Sputum copper was higher in all disease groups vs control but only reached statistical significance for CF ($P < .01$).

In some subjects trace element levels were below the limit of detection of the assay, in particular for manganese. Interestingly, zinc and iron were detectable in all subjects with CF. Please refer to Table 2 for further information.

Correlation of Sputum Zinc and Iron Levels With Lung Function, Sputum Cytology, and Sputum Biomarkers in Patients With CF

As the most statistically significant changes were seen in the CF group, further comparisons were made with clinical data and other inflammatory markers for this group. Sputum zinc and iron levels were correlated in patients with CF (Spearman $r = 0.75$, $P < .05$, data not shown). There was a negative correlation of zinc and FEV_1% predicted in the CF group (Spearman $r = -0.469$, $P < .05$, Fig 2A). Sputum iron was also negatively correlated with FEV_1% (Spearman $r = -0.43$, $P < .05$, data not shown). Sputum neutrophil % and zinc levels were correlated (Spearman $r = 0.67$, $P < .05$, data not shown). Zinc

Table 2—*Levels of Metal Elements (μg/L) of Processed Sputum Supernatant for Zinc, Iron, Manganese, and Copper*

Disease	Zinc	Iron	Manganese	Copper
Control	15.35 (10.4-25.6)	13.5 (8.6-21.5)	0 (0-0.25)	8.6 (3-16.4)
Asthma	12.7 (7.2-41.4)	30.0 (6.9-35.3)	0.8 (0.2-1.7)[a]	15.2 (8.6-29.5)
COPD	25.4 (9.8-50.7)	21.3 (3.1-35.6)	0 (0-0.7)	15.2 (12.2-22)
CF	135.3 (54.2-209.6)[b]	56.9 (24.3-115.3)[a]	0.3 (0.1-0.8)	19.5 (14.5-30.1)[c]
Bronchiectasis	111.3 (46.1-150.7)[b]	54.2 (22.7-91.6)[a]	0.6 (0.2-1.3)[c]	15.7 (10.9-33.3)

Data are displayed as: median (interquartile range). *P* values are displayed for disease group vs control; for *P* values between groups please consult text. Zinc was below the limit of detection of the assay in four control and one COPD. Iron was below the limit of detection in four control, one asthma, and one COPD. Manganese was below the limit of detection in 14 control, three asthma, 13 COPD, six CF, and one bronchiectasis. See Table 1 for expansion of abbreviation.

[a] $P < .01$ vs control.

[b] $P < .001$ vs control.

[c] $P < .05$ vs control.

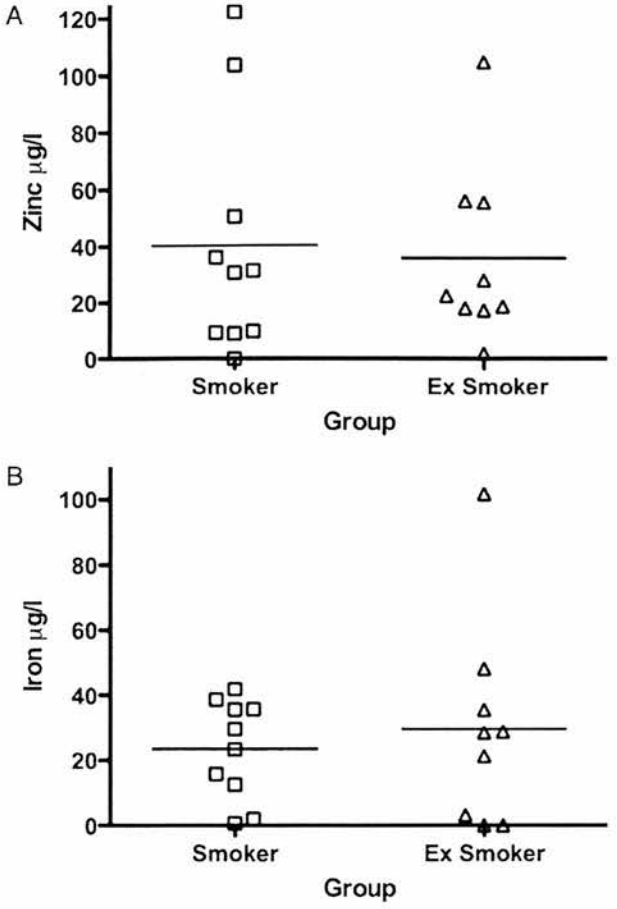


FIGURE 1. Smoking status and sputum trace element level in COPD. Data for zinc (*A*). Data for iron (*B*).

levels were unrelated to the underlying colonizing organism (data not shown).

Sputum zinc and calprotectin levels correlated with high statistical significance (Spearman $r = 0.86$, $P < .001$, Fig 2*B*). Sputum zinc significantly correlated with MPO and IL-8 (Spearman $r = 0.81$, $P < .001$, Spearman $r = 0.67$, $P < .001$, respectively, Figures 2*C* and 2*D*). Sputum iron demonstrated similar but less significant correlations to calprotectin, MPO, and IL-8 (data not shown).

Serial Measurement of Metals in CF Exacerbation

Sputum zinc levels decreased significantly following antibiotic therapy for an exacerbation ($P < .01$; Fig 3). There were no significant serial changes in iron, manganese or copper.

DISCUSSION

Total elemental zinc and iron concentrations are elevated in sputum from subjects with CF and non-CF bronchiectasis compared with healthy control subjects. There is a small degree of overlap between the CF and control groups, but this may be explained by the finding that patients with CF in this small subgroup had better lung function and thus less severe lung disease (data not shown). Sputum zinc levels were also significantly higher in CF and non-CF bronchiectasis compared with COPD. Sputum zinc levels decrease significantly over the course of a CF exacerbation. Zinc is strongly correlated with calprotectin in CF sputum as well as with other inflammatory markers, such as IL-8 and MPO.

Protein biomarkers, such as cytokines, have been used in previous studies to assess levels of airways inflammation.[20-22] However, protease activity in expectorated sputum may affect the robustness of cytokine assays.[23] The potential association we have shown between trace element levels and inflammation in sputum samples combined with their likely resistance to degradation commends them as potentially robust markers of lung pathophysiology.

Serum zinc has previously been suggested as a marker of lung disease,[24,25] but we believe this to be the first study to describe an association between sputum zinc and inflammation in individuals with CF lung disease and non-CF bronchiectasis. Furthermore, we demonstrate its potential use as a serial marker during treatment of an exacerbation, although this must be interpreted with caution as in two subjects zinc levels actually increased, whereas in four others there was only a modest decrease of levels. Nevertheless, the majority of subjects demonstrated a decrease with treatment and as such further investigation is clearly merited.

We thus suggest that sputum zinc may be used as a biomarker in suppurative diseases, such as CF. We do realize, however, that as we have used a relatively small sample size, albeit similar to those use in previous biomarker studies, these data represent a novel observation and further validation in larger studies is required. Furthermore, a parallel measure of zinc in serum may have added further insight into the functional significance of these findings, as would knowledge of individual subject dietary zinc intake.

Zinc homeostasis may play an important role in modulating the immune response to inflammation, with high concentrations of zinc inducing peripheral blood monocyte apoptosis[26] and promoting cytokine production.[27] Conversely, low concentrations of zinc may suppress monocyte function and decrease neutrophil phagocytosis.[28] Zinc may also interact with the airway epithelium.[29,30] For example, zinc deprivation of bronchial epithelial cells in culture induces apoptosis,[31,32] and zinc deficiency in a murine model of asthma induces epithelial cell apoptosis and airways inflammation.[33] We have measured total zinc content of sputum (ie, bound and unbound), whereas

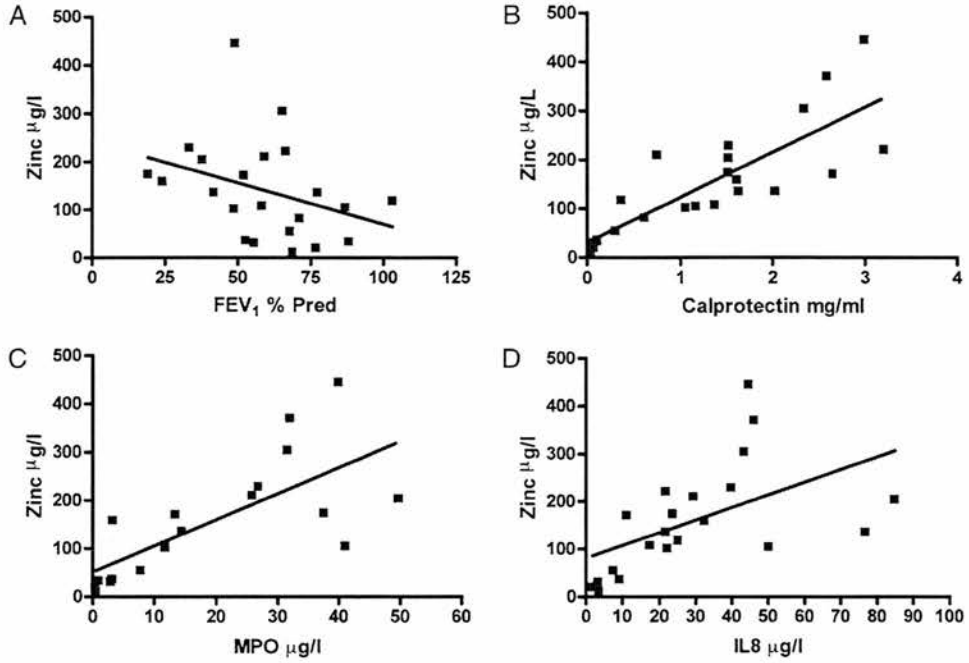


FIGURE 2. Correlation of sputum zinc with biomarkers of lung inflammation and physiology. Correlation between FEV_1% predicted and sputum zinc (Spearman $r = -0.47$, $P < .05$) (*A*). Correlation between sputum calprotectin and zinc. (Spearman $r = 0.86$, $P < .001$) (*B*). Correlation between sputum MPO and zinc (Spearman $r = 0.81$, $P < .001$) (*C*). Correlation between sputum IL-8 and zinc (Spearman $r = 0.67$, $P < .001$) (*D*). IL = interleukin; MPO = myeloperoxidase.

the amount of freely available unbound zinc might be equally important. The excess of zinc in sputum is, however, a possible explanation for the low serum zinc levels in subjects with CF compared with healthy controls.[34]

Increases in sputum iron have been described in CF[9-12] and exceed the levels observed in COPD.[12] Iron is a prerequisite for microbial growth, with increased levels in CF sputum possibly contributing to the proliferation of bacteria such as *P aeruginosa*.[11] The source of this iron is unclear, with leak from the circulation being suggested, although further work is required to investigate this. Cigarette smoke has also been suggested as a potential source of airways iron,[35] but we have demonstrated no difference in sputum iron between current smokers and ex-smokers with COPD in this study.

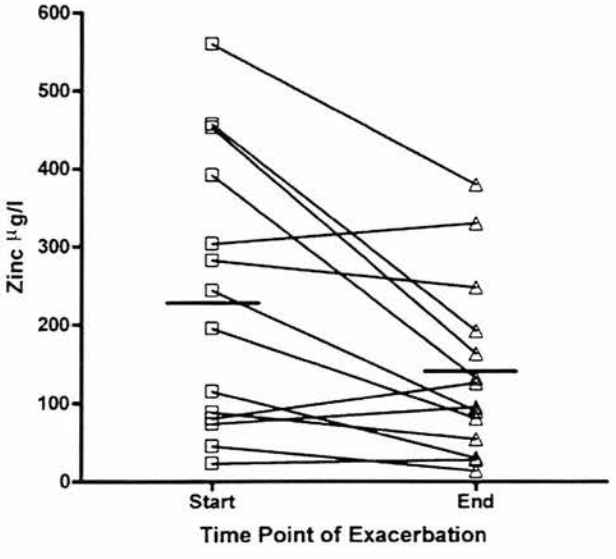


FIGURE 3. Sputum zinc levels in CF exacerbation (n = 14). Sputum zinc decreases over the course of an exacerbation with antibiotic treatment ($P < .01$). Horizontal lines represent means. CF = cystic fibrosis.

Associations between the levels of sputum manganese and copper with disease type are less obvious. Manganese was only significantly elevated in the asthma and non-CF bronchiectasis groups and copper only in CF. Both metals (like zinc) are cofactors for superoxide dismutases (SODs), which have leading roles in alleviating oxidative stress in the lung.[36] Furthermore, the demonstration that manganese differentiated subjects with asthma from control subjects but not subjects with CF from control subjects suggests a possible role for this element in nonsuppurative lung disease. SODs are downregulated in asthmatic airways,[36] suggesting sputum copper and manganese are not merely tracking levels of these mediators. A measurement of SOD level or activity would help to elucidate this complicated relationship but was not performed in this study and thus forms the basis of future work by this group.

Sputum zinc levels correlated with biomarkers of airways inflammation, namely calprotectin, MPO,

and IL-8. Sputum zinc adds to the growing number of potential biomarkers in sputum and may be seen to compliment these as well as offering new insight into pulmonary inflammation. Calprotectin is a highly abundant neutrophil protein found in the CF airway[4,5] with antiinflammatory and proinflammatory functions, and the ability to chelate zinc and other cations.[7,8,37,38] Calprotectin promotes apoptosis in cell lines via the exclusion of zinc,[39,40] and zinc and calprotectin have been demonstrated to colocalize in staphylococcal abscess in a murine model.[8] As such, we would suggest that the interaction of zinc and calprotectin in the CF airway is of mechanistic importance, particularly when we consider that *S aureus* is a major pathogen in early disease.

Elevated levels of calprotectin and zinc may reflect passive release of the neutrophil contents in view of the large number of necrotic neutrophils in the CF airway,[41] or may represent active secretion as is observed with lactoferrin release from neutrophils.[42] A passive release of zinc, on cell necrosis, would be supported by the correlation of zinc with both neutrophil percentage in samples and calprotectin, a cytoplasmic protein in neutrophils. A more controlled active release of zinc may be suggested, however, by the correlation of zinc with MPO, a neutrophil granule protein released from activated neutrophils. Nevertheless, the higher correlation is of zinc, and calprotectin might simply reflect an overall abundance of neutrophils, which are rich in both of these substances,[43,44] as neutrophils contain 5 to 10 ng zinc per 10^6 cells.[43] It is also important to consider that the zinc we have measured in the airway could also be complexed to calprotectin (or other proteins) or could be due to leakage from the pulmonary circulation during inflammation.

In conclusion, we have demonstrated elevated levels of trace metals (zinc in particular) in the fluid phase of sputum from patients with CF and non-CF bronchiectasis compared with patients with asthma or COPD and healthy adult controls. We also demonstrate that the level of zinc in CF sputum decreases over the course of an infective exacerbation and the use of chemically stable markers in noninvasive assays to monitor the course and severity of lung diseases such as CF would clearly be advantageous. Of course a valid biomarker must be highly reproducible and repeatable and, as such, longitudinal studies to evaluate the robustness of sputum trace metal assays of inflammation are required.

Acknowledgments

Author contributions: *Dr Gray:* contributed to the original concept and design of the study, manuscript writing and preparation, and collecting, processing, and analyzing samples.
Dr Duncan: contributed to overseeing trace element analysis and providing intellectual input to the manuscript.
Dr Noble: contributed to collecting, processing, and analyzing samples.
Ms Imrie: contributed to collecting, processing, and analyzing samples.
Dr O'Reilly: contributed to overseeing trace element analysis and providing intellectual input to the manuscript.
Dr Innes: contributed to providing senior mentorship and intellectual input and was involved in manuscript writing.
Dr Porteous: contributed to providing senior mentorship and intellectual input and was involved in manuscript writing.
Dr Greening: contributed to providing senior mentorship and intellectual input and was involved in manuscript writing.
Dr Boyd: contributed to the original concept and design of the study and manuscript writing and preparation.
Financial/nonfinancial disclosures: The authors have reported to *CHEST* the following conflicts of interest: Dr Noble was supported by an unrestricted grant from GlaxoSmithKline. Drs Gray, Duncan, O'Reilly, Innes, Porteus, Greening, and Boyd and Ms Imrie have reported no potential conflicts of interest with any companies/organizations whose products or services may be discussed in this article.
Other contributions: We thank the Cystic Fibrosis Trust in their sponsorship of this study through the UK CF Gene Therapy Consortium. We also thank the Scottish Trace Element and Micronutrient Reference Laboratory, Glasgow Royal Infirmary, UK, for their support. In particular we thank James Gibbons for assisting in the trace element assay.

References

1. Pavord ID, Pizzichini MM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. *Thorax*. 1997;52(6):498-501.
2. McGarvey LP, Dunbar K, Martin SL, et al. Cytokine concentrations and neutrophil elastase activity in bronchoalveolar lavage and induced sputum from patients with cystic fibrosis, mild asthma and healthy volunteers. *J Cyst Fibros*. 2002;1(4):269-275.
3. Kelly MM, Keatings V, Leigh R, et al. Analysis of fluid-phase mediators. *Eur Respir J*. 2002;20(suppl 37):24S-39S.
4. Gray RD, MacGregor G, Noble D, et al. Sputum proteomics in inflammatory and suppurative respiratory diseases. *Am J Respir Crit Care Med*. 2008;178(5):444-452.
5. MacGregor G, Gray RD, Hilliard TN, et al. Biomarkers for cystic fibrosis lung disease: application of SELDI-TOF mass spectrometry to BAL fluid. *J Cyst Fibros*. 2008;7(5):352-358.
6. Lusitani D, Malawista SE, Montgomery RR. Calprotectin, an abundant cytosolic protein from human polymorphonuclear leukocytes, inhibits the growth of Borrelia burgdorferi. *Infect Immun*. 2003;71(8):4711-4716.
7. Clohessy PA, Golden BE. Calprotectin-mediated zinc chelation as a biostatic mechanism in host defence. *Scand J Immunol*. 1995;42(5):551-556.
8. Corbin BD, Seeley EH, Raab A, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science*. 2008;319(5865):962-965.
9. Stites SW, Walters B, O'Brien-Ladner AR, Bailey K, Wesselius LJ. Increased iron and ferritin content of sputum from patients with cystic fibrosis or chronic bronchitis. *Chest*. 1998;114(3):814-819.
10. Stites SW, Plautz MW, Bailey K, O'Brien-Ladner AR, Wesselius LJ. Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with stable cystic fibrosis. *Am J Respir Crit Care Med*. 1999;160(3):796-801.
11. Reid DW, Withers NJ, Francis L, Wilson JW, Kotsimbos TC. Iron deficiency in cystic fibrosis: relationship to lung disease

severity and chronic Pseudomonas aeruginosa infection. *Chest*. 2002;121(1):48-54.
12. Reid DW, Lam QT, Schneider H, Walters EH. Airway iron and iron-regulatory cytokines in cystic fibrosis. *Eur Respir J*. 2004;24(2):286-291.
13. Singh PK, Parsek MR, Greenberg EP, Welsh MJ. A component of innate immunity prevents bacterial biofilm development. *Nature*. 2002;417(6888):552-555.
14. Cooper GL, Louie A, Baltch AL, et al. Influence of zinc on Pseudomonas aeruginosa susceptibilities to imipenem. *J Clin Microbiol*. 1993;31(9):2366-2370.
15. Pizzichini E, Pizzichini MM, Efthimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J*. 1996;9(6):1174-1180.
16. Pin I, Gibson PG, Kolendowicz R, et al. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax*. 1992;47(1):25-29.
17. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax*. 2002;57(10):875-879.
18. Husson MO, Wizla-Derambure N, Turck D, Gosset P, Wallaert B. Effect of intermittent inhaled tobramycin on sputum cytokine profiles in cystic fibrosis. *J Antimicrob Chemother*. 2005;56(1):247-249.
19. Colombo C, Costantini D, Rocchi A, et al. Cytokine levels in sputum of cystic fibrosis patients before and after antibiotic therapy. *Pediatr Pulmonol*. 2005;40(1):15-21.
20. Brightling CE. Clinical applications of induced sputum. *Chest*. 2006;129(5):1344-1348.
21. Ordoñez CL, Kartashov AI, Wohl ME. Variability of markers of inflammation and infection in induced sputum in children with cystic fibrosis. *J Pediatr*. 2004;145(5):689-692.
22. Sagel SD, Kapsner R, Osberg I, Sontag MK, Accurso FJ. Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. *Am J Respir Crit Care Med*. 2001;164(8 pt 1):1425-1431.
23. Pizzichini E, Pizzichini MM, Efthimiadis A, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med*. 1996;154(2 pt 1):308-317.
24. Beeley JM, Darke CS, Owen G, Cooper RD. Serum zinc, bronchiectasis, and bronchial carcinoma. *Thorax*. 1974;29(1):21-25.
25. Andrews GS. Studies of plasma zinc, copper, caeruloplasmin, and growth hormone: with special reference to carcinoma of the bronchus. *J Clin Pathol*. 1979;32(4):325-333.
26. Chang KL, Hung TC, Hsieh BS, et al. Zinc at pharmacologic concentrations affects cytokine expression and induces apoptosis of human peripheral blood mononuclear cells. *Nutrition*. 2006;22(5):465-474.
27. Driessen C, Hirv K, Rink L, Kirchner H. Induction of cytokines by zinc ions in human peripheral blood mononuclear cells and separated monocytes. *Lymphokine Cytokine Res*. 1994;13(1):15-20.
28. Ibs KH, Rink L. Zinc-altered immune function. *J Nutr*. 2003;133(5)(suppl 1):1452S-1456S.
29. Zalewski PD. Zinc metabolism in the airway: basic mechanisms and drug targets. *Curr Opin Pharmacol*. 2006;6(3):237-243.
30. Zalewski PD, Truong-Tran AQ, Grosser D, Jayaram L, Murgia C, Ruffin RE. Zinc metabolism in airway epithelium and airway inflammation: basic mechanisms and clinical targets. A review. *Pharmacol Ther*. 2005;105(2):127-149.
31. Bao S, Knoell DL. Zinc modulates airway epithelium susceptibility to death receptor-mediated apoptosis. *Am J Physiol Lung Cell Mol Physiol*. 2006;290(3):L433-L441.
32. Carter JE, Truong-Tran AQ, Grosser D, Ho L, Ruffin RE, Zalewski PD. Involvement of redox events in caspase activation in zinc-depleted airway epithelial cells. *Biochem Biophys Res Commun*. 2002;297(4):1062-1070.
33. Truong-Tran AQ, Ruffin RE, Foster PS, et al. Altered zinc homeostasis and caspase-3 activity in murine allergic airway inflammation. *Am J Respir Cell Mol Biol*. 2002;27(3):286-296.
34. Krebs NF, Westcott JE, Arnold TD, et al. Abnormalities in zinc homeostasis in young infants with cystic fibrosis. *Pediatr Res*. 2000;48(2):256-261.
35. Nelson ME, O'Brien-Ladner AR, Wesselius LJ. Regional variation in iron and iron-binding proteins within the lungs of smokers. *Am J Respir Crit Care Med*. 1996;153(4 pt 1):1353-1358.
36. Kinnula VL, Crapo JD. Superoxide dismutases in the lung and human lung diseases. *Am J Respir Crit Care Med*. 2003;167(12):1600-1619.
37. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes [review]. *J Leukoc Biol*. 1993;53(2):197-204.
38. Isaksen B, Fagerhol MK. Calprotectin inhibits matrix metalloproteinases by sequestration of zinc. *Mol Pathol*. 2001;54(5):289-292.
39. Ghavami S, Kerkhoff C, Los M, Hashemi M, Sorg C, Karami-Tehrani F. Mechanism of apoptosis induced by S100A8/A9 in colon cancer cell lines: the role of ROS and the effect of metal ions. *J Leukoc Biol*. 2004;76(1):169-175.
40. Yui S, Nakatani Y, Hunter MJ, Chazin WJ, Yamazaki M. Implication of extracellular zinc exclusion by recombinant human calprotectin (MRP8 and MRP14) from target cells in its apoptosis-inducing activity. *Mediators Inflamm*. 2002;11(3):165-172.
41. Watt AP, Courtney J, Moore J, Ennis M, Elborn JS. Neutrophil cell death, activation and bacterial infection in cystic fibrosis. *Thorax*. 2005;60(8):659-664.
42. Jurado RL. Iron, infections, and anemia of inflammation. *Clin Infect Dis*. 1997;25(4):888-895.
43. Milne DB, Ralston NV, Wallwork JC. Zinc content of cellular components of blood: methods for cell separation and analysis evaluated. *Clin Chem*. 1985;31(1):65-69.
44. Voganatsi A, Panyutich A, Miyasaki KT, Murthy RK. Mechanism of extracellular release of human neutrophil calprotectin complex. *J Leukoc Biol*. 2001;70(1):130-134.

ELSEVIER

Journal of Cystic Fibrosis 9 (2010) 193–198

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Original Article

Sputum and serum calprotectin are useful biomarkers during CF exacerbation

R.D. Gray*, M. Imrie, A.C. Boyd, D. Porteous, J.A. Innes, A.P. Greening

School of Molecular and Clinical Medicine, University of Edinburgh, Western General Hospital, Edinburgh, UK

Received 1 October 2009; received in revised form 25 November 2009; accepted 20 January 2010
Available online 17 March 2010

Abstract

Background: Adequate monitoring of cystic fibrosis lung disease is difficult. CF exacerbation offers a unique setting to test the utility of biomarkers in the assessment of changing airways inflammation. We hypothesised that levels of calprotectin in sputum (and serum) would change informatively following treatment of an exacerbation.

Methods: 27 patients with CF were recruited at onset of pulmonary exacerbation. Sputum and serum were collected at the start and end of antibiotic therapy. Sputum calprotectin, interleukin-8 (IL8), and myeloperoxidase (MPO) were measured, as were serum calprotectin, CRP and vascular endothelial growth factor (VEGF).

Results: Sputum calprotectin decreased following treatment of an exacerbation ($p<0.05$), and was superior to other sputum markers. Serum calprotectin, CRP, and VEGF also decreased significantly ($p=0.002$, $p=0.002$, $p=0.013$ respectively). Serum calprotectin level following treatment had predictive value for time to next exacerbation ($p=0.032$).

Conclusions: This study demonstrates the superiority of calprotectin (in sputum and serum) as a biomarker of CF exacerbation over better-established markers.

1. Introduction

Cystic fibrosis (CF) lung disease is characterised by chronic bacterial infection which begins in early childhood, is persistent throughout life and rapidly evolves to evade host defence systems [1]. Patients with CF experience recurrent episodes of increasing pulmonary symptoms, termed exacerbations, which are accompanied by a decrease in lung function [2]. Viral infections, including respiratory syncitial virus, may initiate pulmonary exacerbation [3] but an increase in the density of colonising organisms [4] or the acquisition of new pathogenic organisms [5] are also important and as such antibiotic therapy decreases the bacterial density of respiratory secretions [6,7].

A major difficulty in studying the aetiology and pathophysiology of CF exacerbations is the lack of consensus for diagnostic criteria despite a definite clinical need [8]. Exacerbation has been defined in major CF therapeutic trials from empirical data [9–11]. Nevertheless in routine practice clinical judgement and changes in lung function are most commonly used to dictate the need for therapy [11]. Irrespective of definition, CF exacerbation represents an *in vivo* state of increasing inflammation in CF lung disease.

Sputum obtained from CF subjects contains a mixture of proteins which may serve as objective measures of lung inflammation. Interleukin 8 [IL-8] [11–16], myeloperoxidase [MPO] [17–20], matrix metaloproteinase 9 [MMP-9] [21] and neutrophil elastase [NE] [13,16,22] have all been advocated and studied. NE and IL-8 correlate inversely to lung function, suggesting a relationship of sputum markers to disease severity [23]. We have recently described calprotectin (also known as calgranulin A/B, S100A8/A9, MRP8/14, CF antigen) in BALF [24] and sputum [25] as a biomarker of CF lung disease. Calprotectin was first described in the serum of CF patients in 1975[26], and later became known as CF antigen[27]. In spite of being present in CF lung secretions in high concentrations, the function of calprotectin in the CF lung and its mechanism of action have yet to be explored. Calprotectin is highly abundant in neutrophils, has pro-inflammatory properties via activation of

* Corresponding author.
E-mail address: r.d.gray@ed.ac.uk (R.D. Gray).

1569-1993/$ - see front matter © 2010 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jcf.2010.01.005

TLR-4[28], and has been demonstrated as central to lung inflammation in non-CF models of lung infection[29].

Sputum protein profiles [17] and cytokine levels such as IL-8 have been demonstrated to change following treatment of CF exacerbations with antibiotic therapy [6,7], although this is not a consistent finding in all studies[30]. Altering the level of bacterial burden in the lung with antibiotic therapy may alter the inflammatory milieu. Thus we may use this model to study the clinical significance of new markers of CF lung disease. Other groups have used exacerbation in CF to demonstrate the presence of novel biomarkers, for example prostaglandin E_2 and cys-leukotrienes, mediators of oxidative stress, were elevated in CF exacerbation compared to stable CF [31] as was HMGB1[32], although these studies did not utilise serial samples in the same patients. Serial monitoring has been used for serum vascular endothelial growth factor, which appears to be a marker of inflammatory change following treatment of CF exacerbation with antibiotic therapy [33].

On the basis of our previous work with calprotectin [24,25] we hypothesised that calprotectin (sputum and serum) would change informatively following treatment of CF exacerbation. We wished to compare its utility to previously assessed biomarkers, sputum IL-8 and serum CRP and VEGF.

2. Methods

The Lothian Hospitals Ethics Committee granted approval for this study. Patients were recruited at the time of a pulmonary exacerbation requiring antibiotics, as determined by the patient's physician on the basis of increased breathlessness, increased sputum production and a decrease from baseline FEV_1. Sputum and serum were collected for the assessment of biomarkers within 24 hrs of commencing treatment with antibiotics and again at cessation. This ranged from one to three weeks of therapy. FEV_1 was recorded at these time points.

Sputum was processed within 2 hours of collection as described previously [34]. In brief, sputum plugs were harvested and processed with 4x weight/volume 0.1% dithiothreitol (DTT) after which 4x weight/volume PBS was added. Samples were filtered through 48 μm mesh and centrifuged at 1200 rpm to remove the cells. Supernatant was stored at -80 °C until further analysis. The cell pellet was re-suspended in PBS, cytospins prepared and stained with May-Grunwald-Giemsa for differential cell counting. All counts were expressed as percentage of the population counted. All samples utilised in the study contained <40% squamous cells ensuring samples were from the lower airway.

Table 1
Patient Demographics, colonising organism and antibiotic treatment.

Patient	M/F	Genotype	Age	FEV1% Pred Start	FEV1% Pred End	Colonising Organism	Treatment For Exacerbation
1	f	ΔF508/3659ΔC	20	67	66	BC, SM, HI, SA	TO, CFZ
2	f	ΔF508/ΔF508	30	42	49	PA, SA	TO, CFZ
3	f	ΔF508/ΔF508	20	13	13	PA	CO, MER
4	f	ΔF508/ΔF508	18	55	60	PA	TO, CFZ
5	f	ΔF508/G551D	18	23	39	BC	UnK
6	m	ΔF508/Unk	46	36	38	PA, SM	TO, CFZ
7	f	ΔF508/ΔF508	21	56	59	PA, SA	TO, MER
8	m	ΔF508/ΔF508	18	58	59	MRSA, SA	TO, CFZ
9	f	ΔF508/G551D	31	31	49	PA	CFZ, CIP
10	m	ΔF508/ΔF508	18	33	43	SA, SM	TAZ, MIN
11	f	ΔF508/ΔF508	20	60	73	PA, Asp	TO, MER
12	m	ΔF508/Unk	32	75	88	PA	CIP, AZI
13	f	ΔF508/P67L	27	45	49	SA, BMV	TO, CFZ
14	f	ΔF508/G542X	23	50	82	SA, HI	TO, CFZ
15	f	ΔF508/ΔF508	22	66	75	PA, SA	CO, CFZ
16	f	ΔF508/Unk	17	38	44	SA	FL, COAMOX
17	m	ΔF508/G542X	22	56	52	PA	TO, MER, AZ
18	m	ΔF508/G551D	41	24	31	SM, PA, SA. Asp	TO, MER
19	m	ΔF508/ΔF508	24	41	49	SA, PA, SM	TO, CFZ
20	m	ΔF508/ΔF508	37	21	27	BC, PA	TO, MER
21	f	ΔF508/ΔF508	22	45	52	PA	TO, MER
22	f	ΔF508/ΔF508	18	15	15	PA, SA	AZ, MER
23	m	ΔF508/G551D	20	28	28	PA	TO, CFZ
24	f	ΔF508/ΔF508	26	61	64	PA, SM, Asp	TO, CFZ
25	m	ΔF508/ΔF508	17	23	28	PA	CO, MER
26	m	ΔF508/ΔF508	17	33	45	PA, SA	TO, CFZ
27	m	ΔF508/3849+10 kb C→T	22	34	49	PA	TO, CFZ

Unk = unknown.
Colonising organisms relate to most recent sputum culture prior to exacerbation recorded for each patient. PA=*Pseudomonas aeruginosa*, BC=*Burkholderia cenocepacia*, BMV=*Burkholderia multivorans*, SA=*Staph aureus*, SM=*Stenotrophomonas maltophillia*, HI=*Haemophilus influenzae*, Asp=*Aspergillus fumigatus*. Treatment for exacerbation was with intravenous antibiotics apart from subject 12 who received oral treatment. AZ=aztreonam, AZI=azithromycin, CIP=ciprofloxacin, CFZ=ceftazidime, CO=colomycin, COAMOX=coamoxiclav, FL=flucloxacillin, MER=meropenem, MIN=minocycline, TAZ=tazobactam/pipericillin, TO=Tobramycin.

Blood was collected into serum tubes with pre-added clotting activator (Monovette serum collection tubes, Sarstedt AG and Co, Germany). The tube was then mixed by inverting 5 times. Blood was left to clot at room temperature for 45 minutes. Tubes were centrifuged at 1800×*g* for 15 minutes at room temperature. Separated serum was removed into cryovials (Nunc, Thermo Fisher Scientific, Denmark) as above and stored at -80 °C until further analysis. A separate EDTA blood sample was taken for routine haematology (white cell count).

Calprotectin was measured in sputum and serum by a double antibody sandwich ELISA, using monoclonal and polyclonal antibodies against human calprotectin complex (gift of Erling Sundrehagen, Norway). Interleukin 8 (Biosource, UK); myeloperoxidase (Assay Designs, Michigan, USA); CRP; and VEGF (Quantikine, R and D Systems, Oxford, UK); were measured using commercial kits according to the manufacturers' instructions. All standard curves and dilutions for sputum ELISAs were performed in the presence of 0.05% DTT to ensure accurate measurement of mediators in sputum as samples had been processed with DTT.

2.1. Prediction of future exacerbations

To investigate whether serum calprotectin at the end of exacerbation could predict patient outcome the clinical case notes were reviewed 1 year following completion of the study and the time to next exacerbation calculated in days. A cut off 9.1 μg/ml (median value in stable non-exacerbating CF subjects) was employed and this divided the group into 13 (<9.1 μg/ml) and 12 (>9.1 μg/ml) patients. The same analysis was performed for CRP using a cut off level of 10 mg/ml (upper limit of normal).

2.2. Statistical analyses

Data analyses were performed with GraphPad Prism software (GraphPad, La Jolla, Ca, USA). Normally distributed data were analysed by paired t test and non-normally distributed data by Wilcoxon sign rank test. Kaplan Meier curves were compared by log rank (Mantel Cox) testing.

3. Results

Twenty-seven patients completed the study (demographics in Table 1). FEV_1 improved over the course of an exacerbation, increasing from 41.8 (SEM 3.2) to 49.1 (3.6)% predicted (p=0.001). Whole blood white cell count decreased from 11.8 (SEM 0.9) to 9.0 (1.5) (p=0.004) and sputum neutrophils from 98.8% to 97.5% (p=0.04), see Table 2.

3.1. Sputum results

Due to limitations in sputum sample size not all patients could be assessed for all biomarkers (priority was given to sputum calprotectin which was measured in all 27 paired samples). There was a significant reduction in the level of calprotectin from median 619.4 (IQ range; 484.1- 971.9)μg/ml to 274.4 (184.0-570.9)μg/ml (p=0.013; Fig. 1). Sputum IL8 and MPO were measured in 26 paired samples (Table 2). Sputum IL8 showed a trend to decrease following treatment, from median 30.8 (18.8-53.5)ng/ml to 20.6 (10.3-60.6)ng/ml (p=0.11). Sputum MPO showed a trend to decrease following treatment, from median 41.3 (18.6-49.8)μg/ml to 24.4 (8.8-45.5)μg/ml (p=0.07).

Table 2
Measurements taken at the start and end of exacerbation treatment.

Measurement	Start of Exacerbation	End of Treatment
FEV_1% predicted	41.8(3.2)	49.1(3.6)**
Sputum Calprotectin μg/ml	619.4 (484.1- 971.9)	274.4 (184.0-570.9)*
Sputum IL8 ng/ml	30.8(18.8-53.4)	20.6(10.3-60.5)
Sputum MPO μg/ml	41.3(18.6-49.8)	24.4(8.8-45.5)
Sputum Neutrophil %	98.8(97.2-99.6)	97.5(95.6-98.7)*
WCC 10^9	11.8 (0.9)	9.0(1.5)**
CRP mg/ml	35.6(8.6-75.2)	9.9(3.0-23.5)**
Serum Calprotectin μg/ml	21.5 (13.3-55.5)	9.3 (6.5-18.2)**
Serum VEGF	385 (226- 582)	236 (143- 412)*

Data are displayed as median (IQR) or mean (SEM) depending on normality of distribution. Paired analysis was performed to investigate which markers changed most significantly with treatment, for exact p values please see text.
* p<0.05.
** p<0.01.

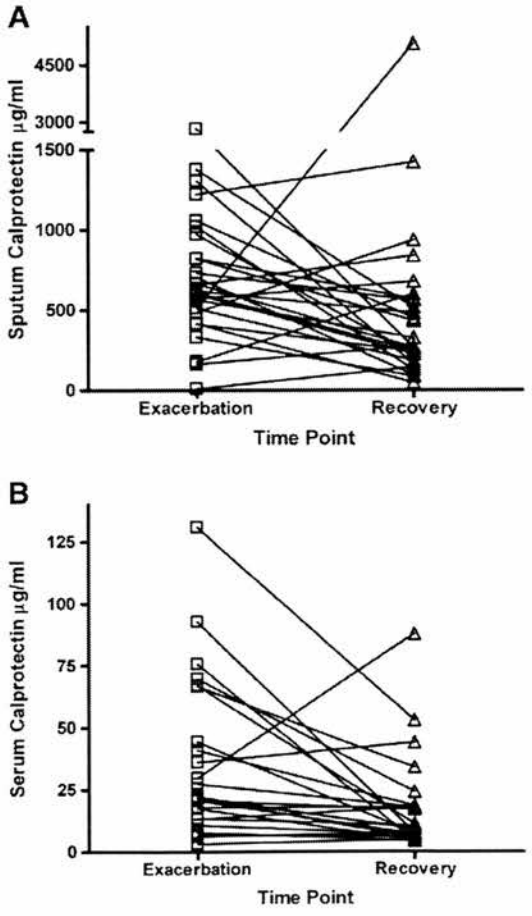


Fig. 1. Panel A. Sputum calprotectin decreases over the course of an exacerbation following treatment with antibiotics; p=0.013. Individual data are shown for 27 subjects. Panel B. Serum calprotectin decreases over the course of an exacerbation following treatment with antibiotics; p=0.002. Individual data are shown for 25 subjects.

3.2. Serum results

Serum was available in 25 patients from 27 recruited as two declined venepuncture. Serum calprotectin decreased from median 21.5 (13.3-55.5) μg/ml to 9.3 (6.5-18.2) ($p=0.002$; Fig. 1). Serum CRP decreased from median 35.6 (8.7-92.0) mg/ml. to 9.9 (3.0-23.5) ($n=22$ paired samples [3 single samples were above the limit of detection of the assay: 300 mg/ml]; $p=0.002$; Table 2). Serum VEGF decreased from median 385 (226- 582) pg/ml to 236 (143- 412) ($p=0.013$; Fig. 2).

3.3. Significant correlations

Serum calprotectin was negatively correlated with FEV_1 (Spearman r -0.49 [$p<0.012$] pre-treatment vs. -0.38 [$p=0.056$] post-treatment), giving an overall Spearman r of -0.48 ($p=0.0004$) for calprotectin and FEV_1 before and after exacerbation treatment. Serum CRP correlated less well with lung function (Spearman r -0.32 [$p=0.12$] pre-treatment vs. -0.26 [$p=0.21$] post-treatment), giving an overall Spearman r of -0.36 ($p=0.011$) for CRP and FEV_1 before and after exacerbation treatment. Sputum calprotectin did not significantly correlate with lung function.

3.4. Predictive values of serum markers

The median time to exacerbation in patients with calprotectin >9.1 μg/ml was 70 days compared to 112 days in the <9.1 μg/ml group ($p=0.032$; Fig. 4). Three patients in the >9.1 μg/ml group died within 18 months of their final study visit. CRP failed to show a difference in the median time to next exacerbation 81 (<10 mg/ml) vs. 84 (>10 mg/ml) days ($p=0.12$; Fig. 3).

4. Discussion

We have demonstrated that treatment of an exacerbation with antibiotic therapy in CF results in decreasing levels of sputum and serum calprotectin. Serum CRP and VEGF also decreased. We have also demonstrated a predictive value of serum calprotectin at the end of exacerbation treatment for time to next exacerbation.

Sputum calprotectin decreased following treatment of a CF exacerbation. We have previously demonstrated high levels of calgranulins A and B (the constituent subunits of calprotectin), by mass spectrometry, in CF sputum and BALF[24,25]. Calprotectin may be secreted from stimulated neutrophils [35], or released at cell death [36] and as such is an appropriate marker for inflammation in the CF airway. Faecal calprotectin has been recognised as a marker of organic bowel disease [37] and can differentiate inflammatory bowel disease, which is neutrophil predominant, from irritable bowel syndrome [38]. Calprotectin may play an important mechanistic role in the CF airway and has been previously implicated in early lung disease in animal models [39]. Furthermore functional knock out of calprotectin in a murine model of pneumonia leads to decreases in inflammatory cell recruitment suggesting an integral role in inflammatory cell recruitment [29]. Thus the change in sputum calprotectin following antibiotic therapy implies a direct association of calprotectin with a changing state of airways inflammation. The exploration of a possible role of calprotectin as a pro-inflammatory molecule in the lung requires further work.

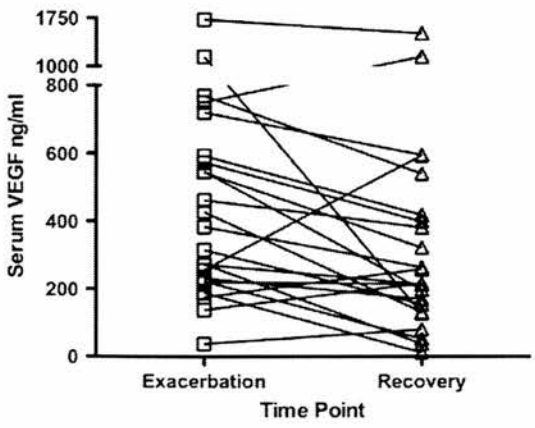


Fig. 2. Serum VEGF decreases over the course of an exacerbation following treatment with antibiotics; $p=0.013$. Individual data are shown for 23 subjects.

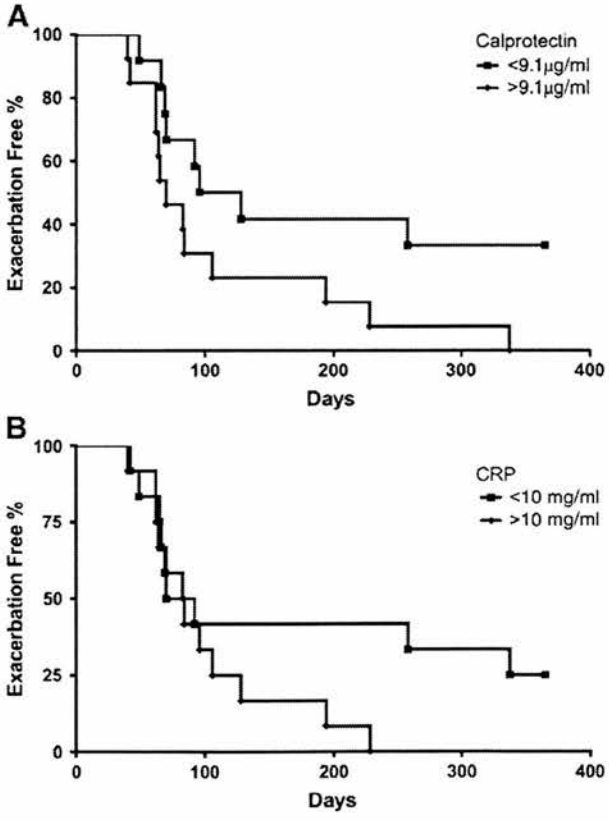


Fig. 3. Panel A. Subjects with serum calprotectin of 9.1 mg/ml at the end of exacerbation treatment have longer time to next exacerbation with median time to exacerbation 112 days vs. 70 days ($p=0.032$). Panel B. Serum CRP of <10 mg/ml did not differentiate time to next exacerbation with median time to exacerbation of 81 vs. 83.5 days ($p=0.125$).

In this study we failed to demonstrate a significant change in sputum IL-8 or MPO following antibiotic therapy, although there were trends to reduction. Decreases in sputum IL-8 following IV antibiotic treatment have been described [6,7], with similar findings reported following nebulised antibiotic therapy [40]. Our study was performed using spontaneously expectorated sputum in line with both Colombo et al [7] and Husson et al [40], but in contrast to Ordonez et al [6], who induced sputum. We do not believe our study was underpowered to demonstrate a change in sputum IL-8 (a secondary outcome), as other groups have demonstrated changes in sputum IL-8 with similar sized patient cohorts [7,40]. The largest study demonstrating changes in sputum IL-8 following antibiotic therapy was performed by Ordonez et al, utilising 42 paired samples and demonstrating a modest decrease in sputum IL-8 (0.5+/- 1.3 log10 pg/ml) [6]. Therefore even if our study was underpowered to detect changes in IL-8 (which we feel unlikely) we have clearly demonstrated the superiority of sputum calprotectin measurement in this population. One possible explanation for our failure to demonstrate a decrease in IL-8, is that our study utilised an adult population with more severe disease compared to Ordonez et al who excluded patients with an FEV_1 of less than 40% [6]. Indeed Downey et al demonstrated no serial change in sputum IL-8 in CF adults following exacerbation treatment, further underlining the possibility that sputum IL-8 is not as powerful a marker in the older patient group[30]. This suggests that IL-8 is a less reliable marker in patients with more advanced lung disease and is consistent with the finding that sputum IL-8 is less well correlated to lung function that other sputum markers such as free elastase [23].

Sputum MPO has been less well studied. As a neutrophil protein we might have expected a change in sputum concentrations following treatment of an exacerbation, and it has been described at high levels in CF sputum compared to control populations [17–20]. The failure to demonstrate a significant change in MPO may be explained by many of the points pertinent to IL-8. And our findings are consistent with a study of oral macrolide antibiotics in CF patients infected with *P. aeruginosa,* which demonstrated no change in sputum MPO following treatment [41]. Also, MPO is a primary granule protein in the neutrophil and as such we could postulate its release from neutrophils may be more tightly controlled than that of calprotectin, a cytoplasmic protein.

Serum calprotectin decreased over the course of an exacerbation. This finding was of higher statistical significance than calprotectin in sputum suggesting less variability in serum sampling than sputum. Calgranulin A (sub-unit of calprotectin) has previously been described in the serum of homozygotes and heterozygotes with CF mutations as *CF antigen* [26]. The serum levels of calprotectin are approximately 4 fold less than those observed in sputum, suggesting that the high concentrations of calprotectin observed in sputum are likely to arise locally in the airways from neutrophils, in particular from necrotic neutrophils, which are more prevalent in the sputum of CF patients with gram negative infection [42]. Changing levels of calprotectin in serum may reflect increased neutrophil recruitment from the bone marrow or leak of calprotectin from the lungs into the systemic circulation due to a breakdown in epithelial barrier integrity although further work is required to investigate this.

Serum CRP and VEGF also decreased significantly. This may have been anticipated as CRP is an acute phase protein previously recognised to change in CF exacerbations [7]. Serum VEGF has also been demonstrated to decrease with treatment of a CF exacerbation, with the main source being postulated as hypoxic lung tissue [33]. In our study both serum CRP and VEGF fell, consistent with previous studies. Serum CRP was demonstrated to show a similar serial change to serum calprotectin following exacerbation treatment but was less well correlated to lung function suggesting a more significant relationship of serum calprotectin to the airway than CRP. However this study does suggest a role for the measurement of CRP in the clinical management of CF exacerbations.

Serum calprotectin concentrations of <9.1 μg/ml at the end of an exacerbation predicted a delayed time to next exacerbation, with the median time being 112 days vs. 70 days for patients with calprotectin >9.1 μg/ml. Indeed three patients in low serum calprotectin group had not exacerbated by 1 year, whereas 3 patients in the high group had died by the time of follow up. CRP was less good in this regard, with no difference in median time to next exacerbation between those patients with normal and those with raised CRP values at the end of exacerbation. Further studies are now required to assess and validate serum calprotectin as a predictor of outcome in CF, but the current data raise the possibility that calprotectin levels may inform whether treatment needs to be prolonged.

We conclude that sputum and serum calprotectin decrease significantly with treatment of an exacerbation and are superior to sputum IL8 and serum CRP and VEGF, all of which have been advocated hitherto, as indicators of response. The additional value of a serum biomarker is recognised because of the greater ease of sample acquisition and processing. Further investigation is required to assess the potential clinical impact of these novel observations.

Role of the funding source

RDG and ACB were funded by the Cystic Fibrosis Trust. RDG was supported in part by the Medical Research Council (Programme Grant G9313618). The funding source had no input into the design, implementation or analysis of the study.

Conflict of interest statement

None of the authors have any conflict of interest with regards to this manuscript.

References

[1] Boucher RC. New concepts of the pathogenesis of cystic fibrosis lung disease. Eur Respir J January 1 2004;23(1):146–58.
[2] Goss CH, Burns JL. Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. Thorax Apr 2007;62(4):360–7.

[3] Hiatt PW, Grace SC, Kozinetz CA, Raboudi SH, Treece DG, Taber LH, et al. Effects of viral lower respiratory tract infection on lung function in infants with cystic fibrosis. Pediatrics Mar 1999;103(3):619–26.
[4] Aaron SD, Ramotar K, Ferris W, Vandemheen K, Saginur R, Tullis E, et al. Adult cystic fibrosis exacerbations and new strains of Pseudomonas aeruginosa. Am J Respir Crit Care Med Apr 1 2004;169(7):811–5.
[5] Smith AL, Redding G, Doershuk C, Goldmann D, Gore E, Hilman B, et al. Sputum changes associated with therapy for endobronchial exacerbation in cystic fibrosis. J Pediatr Apr 1988;112(4):547–54.
[6] Ordonez CL, Henig NR, Mayer-Hamblett N, Accurso FJ, Burns JL, Chmiel JF, et al. Inflammatory and microbiologic markers in induced sputum after intravenous antibiotics in cystic fibrosis. Am J Respir Crit Care Med Dec 15 2003;168(12):1471–5.
[7] Colombo C, Costantini D, Rocchi A, Cariani L, Garlaschi ML, Tirelli S, et al. Cytokine levels in sputum of cystic fibrosis patients before and after antibiotic therapy. Pediatr Pulmonol Jul 2005;40(1):15–21.
[8] Marshall BC. Pulmonary exacerbations in cystic fibrosis: it's time to be explicit! Am J Respir Crit Care Med Apr 1 2004;169(7):781–2.
[9] Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, et al. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. N Engl J Med Sep 8 1994;331(10):637–42.
[10] Ramsey BW, Pepe MS, Quan JM, Otto KL, Montgomery AB, Williams-Warren J, et al. Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. Cystic Fibrosis Inhaled Tobramycin Study Group. N Engl J Med Jan 7 1999;340(1):23–30.
[11] Rabin HR, Butler SM, Wohl ME, Geller DE, Colin AA, Schidlow DV, et al. Pulmonary exacerbations in cystic fibrosis. Pediatr Pulmonol May 2004;37(5):400–6.
[12] Sagel SD, Kapsner R, Osberg I, Sontag MK, Accurso FJ. Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. Am J Respir Crit Care Med Oct 15 2001;164(8 Pt 1): 1425–31.
[13] Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. J Pediatr Dec 2002;141(6):811–7.
[14] Salva PS, Doyle NA, Graham L, Eigen H, Doerschuk CM. TNF-alpha, IL-8, soluble ICAM-1, and neutrophils in sputum of cystic fibrosis patients. Pediatr Pulmonol Jan 1996;21(1):11–9.
[15] Henig NR, Tonelli MR, Pier MV, Burns JL, Aitken ML. Sputum induction as a research tool for sampling the airways of subjects with cystic fibrosis. Thorax Apr 2001;56(4):306–11.
[16] McGarvey LP, Dunbar K, Martin SL, Brown V, Macmahon J, Ennis M, et al. Cytokine concentrations and neutrophil elastase activity in bronchoalveolar lavage and induced sputum from patients with cystic fibrosis, mild asthma and healthy volunteers. J Cyst Fibros Dec 2002;1(4):269–75.
[17] Sloane AJ, Lindner RA, Prasad SS, Sebastian LT, Pedersen SK, Robinson M, et al. Proteomic analysis of sputum from adults and children with cystic fibrosis and from control subjects. Am J Respir Crit Care Med Dec 1 2005;172(11):1416–26.
[18] Regelmann WE, Siefferman CM, Herron JM, Elliott GR, Clawson CC, Gray BH. Sputum peroxidase activity correlates with the severity of lung disease in cystic fibrosis. Pediatr Pulmonol Jan 1995;19(1):1–9.
[19] Meyer KC. Neutrophils, myeloperoxidase, and bronchiectasis in cystic fibrosis: green is not good. J Lab Clin Med Sep 2004;144(3):124–6.
[20] Ordonez CL, Kartashov AI, Wohl ME. Variability of markers of inflammation and infection in induced sputum in children with cystic fibrosis. J Pediatr Nov 2004;145(5):689–92.
[21] Sagel SD, Kapsner RK, Osberg I. Induced sputum matrix metalloproteinase-9 correlates with lung function and airway inflammation in children with cystic fibrosis. Pediatr Pulmonol Mar 2005;39(3):224–32.
[22] Downey DG, Martin SL, Dempster M, Moore JE, Keogan MT, Starcher B, et al. The relationship of clinical and inflammatory markers to outcome in stable patients with cystic fibrosis. Pediatr Pulmonol Mar 2007;42(3):216–20.
[23] Mayer-Hamblett N, Aitken ML, Accurso FJ, Kronmal RA, Konstan MW, Burns JL, et al. Association between Pulmonary Function and Sputum Biomarkers in Cystic Fibrosis. Am J Respir Crit Care Med Apr 15 2007;175(8):822–8.
[24] Macgregor G, Gray RD, Hilliard TN, Imrie M, Boyd AC, Alton EW, et al. Biomarkers for cystic fibrosis lung disease: Application of SELDI-TOF mass spectrometry to BAL fluid. J Cyst Fibros Sep 2008;7(5):352–8.
[25] Gray RD, MacGregor G, Noble D, Imrie M, Dewar M, Boyd AC, et al. Sputum proteomics in inflammatory and suppurative respiratory diseases. Am J Respir Crit Care Med Sep 1 2008;178(5):444–52.
[26] Wilson GB, Fudenberg HH, Jahn TL. Studies on cystic fibrosis using isoelectric focusing. I. An assay for detection of cystic fibrosis homozygotes and heterozygote carriers from serum. Pediatr Res August 1975;9(8):635–40.
[27] Dorin JR, Novak M, Hill RE, Brock DJ, Secher DS, Van Heyningen V. A clue to the basic defect in cystic fibrosis from cloning the CF antigen gene. Nature Apr 9-15 1987;326(6113):614–7.
[28] Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, Van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med Sep 2007;13(9): 1042–9.
[29] Raquil MA, Anceriz N, Rouleau P, Tessier PA. Blockade of antimicrobial proteins S100A8 and S100A9 inhibits phagocyte migration to the alveoli in streptococcal pneumonia. J Immunol Mar 1 2008;180(5):3366–74.
[30] Downey DG, Brockbank S, Martin SL, Ennis M, Elborn JS. The effect of treatment of cystic fibrosis pulmonary exacerbations on airways and systemic inflammation. Pediatr Pulmonol Aug 1 2007;42(8):729–35.
[31] Reid DW, Misso N, Aggarwal S, Thompson PJ, Walters EH. Oxidative stress and lipid-derived inflammatory mediators during acute exacerbations of cystic fibrosis. Respirology Jan 2007;12(1):63–9.
[32] Rowe SM, Jackson PL, Liu G, Hardison M, Livraghi A, Solomon GM, et al. Potential Role of High-Mobility Group Box 1 in Cystic Fibrosis Airway Disease. Am J Respir Crit Care Med October 15 2008;178(8): 822–31.
[33] McColley SA, Stellmach V, Boas SR, Jain M, Crawford SE. Serum vascular endothelial growth factor is elevated in cystic fibrosis and decreases with treatment of acute pulmonary exacerbation. Am J Respir Crit Care Med Jun 2000;161(6):1877–80.
[34] Pizzichini E, Pizzichini MM, Efthimiadis A, Hargreave FE, Dolovich J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. Eur Respir J Jun 1996;9(6):1174–80.
[35] Boussac M, Garin J. Calcium-dependent secretion in human neutrophils: a proteomic approach. Electrophoresis Feb 2000;21(3):665–72.
[36] Voganatsi A, Panyutich A, Miyasaki KT, Murthy RK. Mechanism of extracellular release of human neutrophil calprotectin complex. J Leukoc Biol Jul 2001;70(1):130–4.
[37] Fagerhol MK. Calprotectin, a faecal marker of organic gastrointestinal abnormality. Lancet Nov 25 2000;356(9244):1783–4.
[38] Costa F, Mumolo MG, Ceccarelli L, Bellini M, Romano MR, Sterpi C, et al. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. Gut Mar 2005;54(3):364–8.
[39] Cohen JC, Larson JE. Pathophysiologic consequences following inhibition of a CFTR-dependent developmental cascade in the lung. BMC Dev Biol 2005;5:2.
[40] Husson MO, Wizla-Derambure N, Turck D, Gosset P, Wallaert B. Effect of intermittent inhaled tobramycin on sputum cytokine profiles in cystic fibrosis. J Antimicrob Chemother Jul 2005;56(1):247–9.
[41] Ordonez CL, Stulbarg M, Grundland H, Liu JT, Boushey HA. Effect of clarithromycin on airway obstruction and inflammatory markers in induced sputum in cystic fibrosis: a pilot study. Pediatr Pulmonol 2001 Jul;32(1):29–37.
[42] Watt AP, Courtney J, Moore J, Ennis M, Elborn JS. Neutrophil cell death, activation and bacterial infection in cystic fibrosis. Thorax Aug 2005;60 (8):659–64.