Exploring the relationship between loss of CFTR protein function and markers of disease severity in chronic suppurative lung disease

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Declaration of Originality

I declare that the work presented in this thesis is my own, unless otherwise stated. Any work or data from other sources has been appropriately acknowledged and referenced

Abstract

Cystic fibrosis (CF) and Primary Ciliary Dyskinesia (PCD) are chronic suppurative lung diseases (CSLD). CF is characterised by inherited mutations affecting the cystic fibrosis transmembrane regulator (CFTR) protein, which is thought to be normal in PCD, however the role of CFTR in disease is incompletely understood. This thesis investigates the relationship between CFTR, inflammation and airway health, firstly in the context of the CF gene therapy Multidose trial followed by contrasting CF, PCD and control patients.

The first study explored the relationship between lower airway potential difference (LAPD) measurements performed in the Multidose trial as a measure of CFTR function, and physiological, radiographic and inflammatory markers of disease severity. At baseline, FEV_1 correlated with basal LAPD measurements, however not between restored chloride secretion and change in airway disease following treatment; implicating the role of sodium transport, not chloride in disease pathogenesis. As no direct correlation was seen, I went on to explore an alternative theory that a bi-directional relationship exists between CFTR and inflammation; CFTR dysfunction triggers a hyper-inflammatory state and inflammation causes secondary CFTR dysfunction.

Cell cultures were cultivated from the nasal epithelium of patients with CF, PCD and controls. Both at baseline and following stimulation with common respiratory pathogens, the levels of inflammatory mediators in the supernatant from each group of cells were comparable. The numbers involved with this study were small, however did not indicate that CF cells cultured in these conditions *(in vitro)* were hyper-inflammatory.

The final study explored *in vivo* whether inflammation causes secondary CFTR dysfunction. Nasal potential difference (NPD) measurements were compared with localised levels of inflammation in subjects with CF, PCD and controls. PCD traces showed reduced chloride secretion, however it was not possible to differentiate secondary CFTR dysfunction from damage to epithelial cell integrity. Elevated levels of inflammatory mediators were detected in PCD nasal fluid, however the results were variable and these levels did not correlate with NPD measurements of ion channel function.

These studies did not support the hypothesis that there is a direct relationship between CFTR function and airway disease, that *in vitro* CF cells are hyperinflammatory, or *in vivo* that inflammation leads to secondary CFTR dysfunction. The experiments performed in this thesis provide a basis for future work exploring this relationship, and may help guide future trials for novel therapies in CF.

Acknowledgments

It was a privilege to work at the Department of Gene Therapy and at the Royal Brompton Hospital during this MD(Res). Both places have been incredible places to work and I will take forward the enthusiasm and dedication of everyone for works there for the rest of my career, in addition to many good friends.

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The generosity of patients with their time never fails to amaze me. Thank you so much to all of them, and I hope that some of the work in this will contribute in some way towards helping CF in the future.

And finally, a big thank you to the cabin crew on TigerAir from Melbourne to Sydney for handing in my laptop, without which this thesis would not have been submitted.

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Abbreviations

Abbreviations for clinical studies

Gene therapy Mutlidose Trial	A randomised, double-blind, placebo-controlled Phase 2B clinical trial of repeated application of gene therapy in patients with cystic fibrosis
The Run-In study	Longitudinal assessment of clinical measurements in patients with cystic fibrosis in preparation for a clinical trial of CFTR gene therapy
The Pilot-Study	Evaluation of safety and gene expression with a single dose of pGM169/GL67A administered to the nose and lung of individuals with cystic fibrosis

Abbreviations used throughout the thesis

AE	Adverse events
Ag/AgCl electrode	Silver/Silver chloride (electrode)
ASL	Airway surface liquid
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CEV	Cumulative expired volume
CF	Cystic fibrosis
CFTR	Cystic Fibrosis transmembrane conductance regulator gene
CFTR	Cystic Fibrosis transmembrane conductance regulator protein
CpG	Cytosine-phosphate-Guanine (nucleotide motifs)

CRF	Clinical research facility
CRP	C-reactive protein
CT	Computerised tomography
CV	Coefficient of variation
DIOS	Distal intestinal obstruction syndrome
DNA	Deoxyribonucleic acid
ENaC	Epithelial sodium channel
FDA	U.S Food and Drug Administration
FEV_1	Forced expiratory volume in 1 second
FRC	Functional residual capacity
FVC	Forced vital capacity
GL67A	Cationic lipid used in clinical programme
GTAC	Gene Therapy Advisory Committee
HRCT	High-resolution computerized tomography
ICM	Intestinal current measurement
IQR	Interquartile range
LAPD	Lower airway potential difference
LCI	Lung clearance index
MBW	Multiple breath washout
MCC	Mucociliary clearance
MHRA	Medicines and Healthcare products Regulatory Agency
MMP	Matrix myeloperoxidase
mRNA	Messenger RNA

NF- kB	Nuclear factor - kB
MDI	Metered dose inhaler
PBS	Phosphate buffered saline
PCD	Primary ciliary dyskinesia
PCL	Periciliary liquid
PD	Potential Difference
NPD	Nasal Potential Difference
SAE	Serious adverse event
SD	Standard deviation
SOP	Standard operating procedure
TNF	Tumour necrosis factor
UKCFGTC	UK Cystic Fibrosis Gene Therapy Consortium
WCC	Total white cell count

Abbreviations used within this thesis for potential difference perfusion solutions

Ringer's	Ringers solution
Ringers/A	Ringers solution with amiloride (0.1mM)
ZCl/A	Zero Chloride solution with amiloride (0.1mM)
ZCl/A/Iso	Zero Chloride solution with amiloride (0.1mM) and isoprenaline (10uM)
ZCl/Iso	Zero Chloride solution with isoprenaline (10uM)

Members of the UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC Strategy group)

RBH	Royal Brompton Hospital
WGH	Western General Hospital

RHSC	Royal Hospital for Sick Children, Edinburgh

Member of the UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC) Strategy Group

Listed according to site

Imperial College	Professor Eric Alton
	Professor Jane Davies
	Professor Uta Griesenbach
	Mrs Tracy Higgins
University of Edinburgh	Dr Chris Boyd
	Dr Alistair Innes
	Professor David Porteous
University of Oxford	Dr Deborah Gill
	Dr Steve Hyde

1 Chapter 1 - Introduction

The cystic fibrosis transmembrane regulator (CFTR) protein is a cAMP-regulated ion channel expressed on the apical surface of epithelial cells. It is clear that within the respiratory tract CFTR plays a crucial role in maintaining airway health. Patients with cystic fibrosis (CF), a disease in which mutations in the *CFTR* gene results in reduced or absent CFTR function suffer recurrent respiratory tract infections leading to chronic airway inflammation and usually terminal lung disease. The mechanisms by which impaired CFTR function leaves the CF airway vulnerable to infection, and the progression to the subsequent infective and inflammatory lung disease are currently incompletely understood. It has recently been proposed that a complex relationship exists between CFTR and inflammation, with studies indicating that the relationship is circular: CFTR dysfunction results in inflammation and inflammatory mediators lead to impaired CFTR function. A better understanding of the link between CFTR dysfunction, inflammation and the progression of lung disease could significantly influence the development of novel therapeutic targets for CF. In addition there could be consequences for patients with other chronic suppurative lung diseases (CSLD), in which CFTR dysfunction secondary to inflammation may contribute to disease progression.

This thesis has explored the complex, possible bi-directional relationship between CFTR protein function and markers of disease severity in CSLD in order to better understand how close the relationship is between the loss of CFTR function and the pathogenesis of inflammatory airway diseases. The introduction will explore what is currently known about why CFTR function is important in airway health and introduce the themes that are developed in subsequent chapters.

1.1 Host defence of the lung: an overview

In order to explore the effect of impaired CFTR protein function on the airway, it is necessary to understand how the airway protects itself from recurrent exposures to infections, allergens and pollutants in the inspired air.

1.1.1 Innate immune response:

The lung possesses an array of innate defence mechanisms designed to detect foreign particles and remove them from the epithelial surface. First line barriers consist of physical and anatomical

mechanisms designed to remove pathogens followed by a vast array of antimicrobial compounds, and pathogen receptors to facilitate elimination from the airway.

1.1.1.1 Mucociliary clearance

The respiratory tract is protected by local mucociliary clearance (MCC), a process considered to be fundamental to the airway's innate defence [1] [2]. The mechanism involves the integration of ciliated epithelium, periciliary fluid and mucus, the latter two components constituting the airway surface liquid (ASL). The mucus acts as a physical and chemical barrier onto which particles and organisms adhere. Once attached, immune cells can act upon the pathogen, assisted by antimicrobial peptides, immunoglobulins and proteins. The collection of cells, bacteria and mucus is then removed from the airway by ciliary beating and either expectorated by coughing, or swallowed [3, 4]. This process is illustrated in Figure 1.1-1.

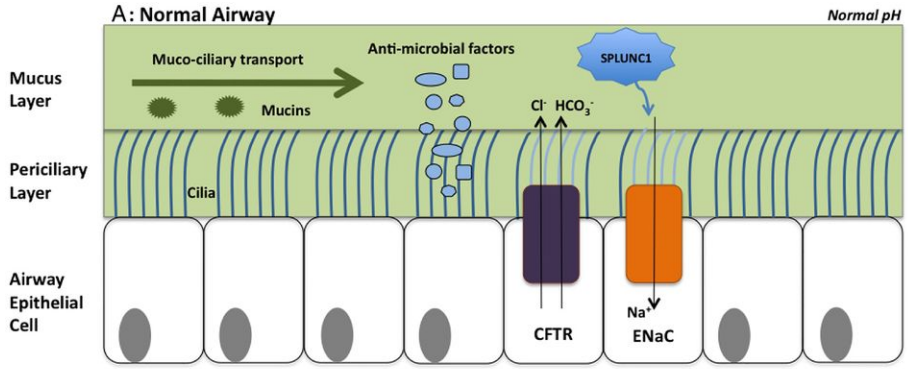


Figure 1.1-1: Diagram of mucus ciliary clearance aiding the elimination of anti-microbial factors in the airway. Reproduced from Brodie et al

Ciliated epithelium

Ciliated cells line parts of the nasal cavity, and from the trachea to the termination of the respiratory bronchioles, interspersed with mucus secreting goblet cells, serous and club cells. Approximately 200 motile cilia project from the cell surface; the ciliary beat is regulated by local signaling resulting in a rapid and coordinated wavelike movement across the epithelial cells. A description of the structure of each individual cilium is covered later in 1.2.1.1.

Epithelial cells first act as a barrier, but are also critically involved in the regulation of both the innate and adaptive immune responses. They play a vital role in the recognition of pathogens, and antimicrobial responses, partly due to secretion of numerous cytokines, discussed below [5].

Airway surface liquid

The ciliated epithelium is lined with periciliary fluid and mucus [6]. The periciliary liquid layer is 5-10 microns deep and the volume is tightly regulated by the epithelial cells as it is critical for effective ciliary beating [3]. Respiratory mucus consists of ions (1%), protein and glycoproteins (mucins) (2-3%), and water (95%), and acts as a visco-elastic gel within which enzymes and immunoglobulins are found. Mucin is produced from goblet cells and submucosal glands. There are a large number of mucin genes, of which at least 12 are expressed in the airway [7]. Once released by the cell, a conformational change occurs and the mucin rapidly expands by hydration. Inhaled particles are entrapped in the mucus layer by the glycoprotein structure. The consistency of the mucus is tightly controlled so as to provide an adhesive but easily transported barrier for pathogens, and is dependent on airway salt concentration, osmolality, water content and ASL pH [8] [9].

1.1.1.2 Soluble factors

Epithelial cells and submucosal glands secrete antimicrobial peptides and proteins into the ASL, which provide an additional layer of protection against invading microorganisms.

Table 1.1-1 lists some of the most abundant factors.

Small antimicrobial peptides
Defensins Cathelicidins
Large antimicrobial peptides
Lysozyme Lactoferrin Palate-lung-nasal-clone (PLUNC) proteins
Proteins
Secretory IgA

Table 1.1-1: List of some of the most abundant soluble factors [10-12].

The composition of the ASL is critical to the function of these antimicrobials, with studies demonstrating that variations in the osmotic and electrolyte balance and ASL pH result in impaired antimicrobial activity and ineffective bacterial killing [13-15]. This is covered in detail in 1.2.3.3.

1.1.1.3 Pattern recognition receptors

Pathogens are detected in the airway epithelium via pattern-recognition molecules (PRMs); epithelial trans-membrane molecules, that bind to pathogen-associated molecular patterns (PAMPs) present on the microorganism. Toll-like receptors (TLRs) are a group of proteins that are PRMs. They are present on airway epithelial cells and play a critical role in the recognition of microorganisms. [16]. TLRs increase transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and regulate the expression of a number of genes, including those encoding adhesion molecules, antimicrobial peptides, and pro-inflammatory cytokines [17].

1.1.2 Inflammatory response

The inflammatory response is the body's response to insults including infection and trauma that are not eliminated by the primary defence barriers. A complex, finely orchestrated interaction occurs between soluble mediators (cytokines) and immune cells to destroy the invading organisms and repair the tissue, consisting of 4 stages: initiation, amplification, phagocytosis and resolution. Acute inflammation in the airway is essential to protect the organ; however excessive inflammation can be harmful and a delicate balance is required.

1.1.2.1 Soluble mediators

Cytokines are secreted polypeptides that are important in cell signaling, and modulating immunity and inflammation. Many cell types release cytokines, including epithelial cells and immune cells and they function via complex interactions. Table 1.1-2 lists the most important cytokines involved in neutrophilic inflammation in the airway. Anti-inflammatory cytokines are crucial to down-regulate the response and include interleukins 6 and 10 (IL-6, IL-10), interleukin-1 receptor antagonist protein (IRAP), transforming growth factor- β (TGF- β) [18].

Cytokines	Actions
IL-1	Primes neutrophils
	Increases adhesion of neutrophils to endothelium
IL-8	Increases chemotaxis of neutrophils to site of inflammation
	Activates neutrophils
	Increases expression of adhesion molecules
TNFα	Increases chemotaxis of neutrophils to site of inflammation
	Increases adhesion of neutrophils to endothelium
	Induces synthesis of chemoattractant neutrophils
	Increases intermediary metabolism
IL-6	Mediates acute-phase reaction
	Matures B-lymphocytes
	Activates T-lymphocytes
IL-10	Inhibits secretion of TNFα and other cytokines
	Inhibits antigen presentation
IRAP	Inhibits IL-1 receptor binding
	Antagonises activities of IL-1

IL-1, interleukin-1; IL-8, interleukin-8; TNFα, tumour necrosis factor-alpha; IL-6, interleukin-6; IL-10, interleukin-10; IRAP, interleukin-1 receptor antagonist protein.

Table 1.1-2: Action of the main pro-inflammatory and anti-inflammatory cytokines in the airway [19-21]

1.1.2.2 Immune cells

There are a large number of diverse cells of the immune system, all of which have vital roles in the inflammatory response. Amongst these include:

- Dendritic cells are antigen-presenting cells (APCs), which identify pathogens via PRMs, which are ingested and processed. These molecules are subsequently presented to resident T cells which induce the immune effector response [18].

- Macrophages, abundant in the lower airways, phagocytose bacteria and release cytokines to recruit neutrophils and other mononuclear cells to the site. They bridge the innate and adaptive immune system, acting as APCs. They phagocytose dead cells and cellular debris; contributing to resolution of inflammation [22].

- Neutrophils are the first cells to be recruited to a site of injury/ infection when stimulated by cytokines, including IL-8 and TNFα, and eliminate microorganisms by both phagocytosis and extracellular microbe elimination. When activated neutrophils release products (DNA, chromatin and granule proteins) which form a neutrophil extracellular traps (NETS) [23] Neutrophils die during the formation of NETS, with the whole process known as NETosis [24]. They also release cytokines, which further amplify the inflammatory reaction by other cell types.

The subsequent adaptive immune response includes antigen-specific defence mechanisms and takes days to develop. [25].

1.1.2.3 Resolution of inflammation

Resolution is an active process and describes the suppression of inflammatory mediator production and removal of acute inflammatory cells, and is an integral part of the physiological response to infection and tissue injury [26] [27]. The most important mediators involved in this process are listed in Table 1.1-3

Mediators involved in resolution of inflammation
T regulatory cells (CD4+CD25+FoxP3+ and CD4+IL-10+ cells)
Cytokines (IL-10, TGFβ and IL-35)
Arachidonic acid derived lipid mediators (lipoxins, resolvins, protectins and maresins) and other lipids such as ceramide
Chemerin (a chemottractant protein) and its receptor Chem R23
Transcription factors (e.g. lung krüppel-like factor)
Proteins (e.g. annexin A1)
Cell surface markers (e.g. CD200 / CD200R - required for macrophage immune homeostasis)

Table 1.1-3: List of mediators involved in resolution of inflammation [27, 29-31]

The innate host defence and the inflammatory response pathways are necessarily complex and finely integrated systems designed to protect the airway from invading pathogens, eliminate them and restore the lung homeostasis.

1.2 Genetic diseases leading to defects in MCC

Genetic diseases affecting either the cilium itself or the ciliary environment, can compromise MCC giving rise to recurrent infections, chronic inflammation and subsequent respiratory disease [3]. This section describes the inherited condition primary ciliary dyskinesia (PCD) and how defects in the ciliary structure lead to the clinical features of the disease. This is followed by an overview of CF, and our current understanding of how defective CFTR impairs MCC in addition to other proposed mechanisms of how loss of CFTR function contributes to airway disease in CF.

1.2.1 Primary ciliary dyskinesia

PCD is an autosomal recessive disorder of motile cilia, affects up to 1 per 10,000-20,000 births (higher in certain ethnic groups) [32, 33]. Mutations in genes encoding proteins involved in the biogenesis, structure, and/or function of motile cilia can result in PCD. Motile cilia are present in the respiratory tract and elsewhere, therefore PCD manifests as a multi-organ disease.

1.2.1.1 Cilia

Cilia are microscopic hair like structures that protrude from the cell body that are constructed from a microtubule cytoskeleton, the axoneme, which is surrounded by a ciliary membrane. There are 3 main groups: non-motile (primary) cilia which function as sensory organelles and coordinate signaling pathways during development and in tissue homeostasis, nodal cilia, which are a unique type of motile cilia expressed on the embryonic node and help determine left-right asymmetry of the body and motile cilia, discussed below [34] [35] [36].

Motile cilia:

Motile cilia line the surface of the respiratory tract, the female reproductive tract and the cerebral ventricles. Figure 1.2-1 illustrates a transverse section of a motile cilium demonstrating the longitudinal microtubules arranged as 9 doublets forming an outer circle around a central pair (called a 9+2 axoneme). The main structural protein of these doublets is tubulin. The structure is maintained by nexin and radial spokes which connect the outer microtubular doublets with a central sheath of protein around the central tubules. Inner dynein arms (IDA) and outer dynein arms (ODA) are attached to the microtubules and it is the dynein that generates the force for the ciliary beating.

The cilium is composed of over 600 proteins in molecular complexes, which combine functions to produce the ciliary beat.

The most commonly identified abnormalities in PCD are defects in the cilia ultrastructure; dynein arm deficiency (ODA/IDA or both), the central tubules and radial spokes Figure 1.2-1.

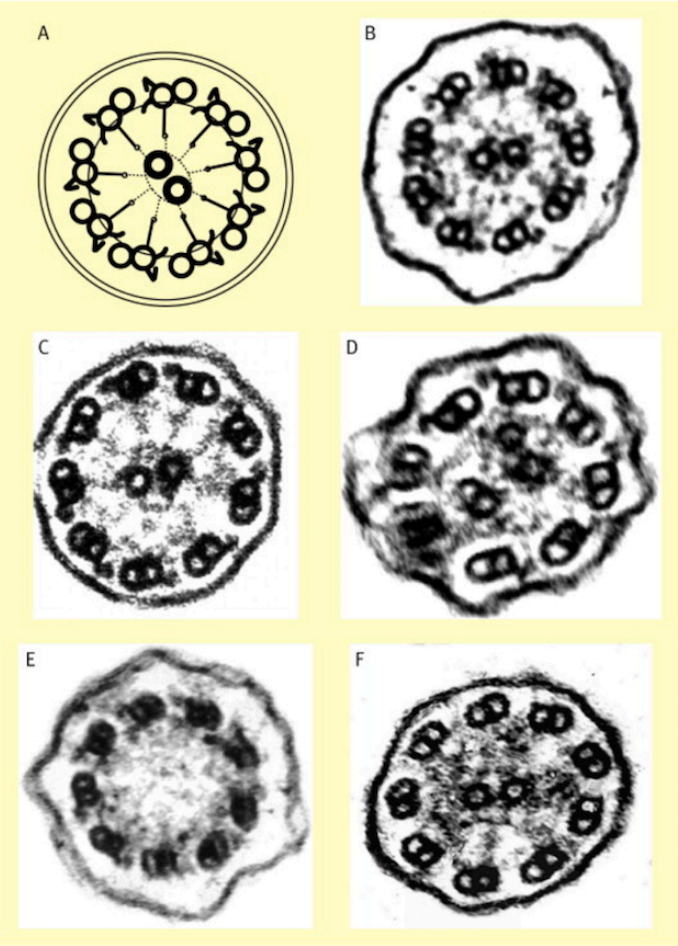


Figure 1.2-1: Common ultrastructural defects found in PCD. (A) Normal ciliary ultrastructural cross section and Transmission electron micrograph of (B) normal ciliary cross-section (C absent inner dynein arms. (D) radial spoke defect. (E) absent central pair found in ciliary transposition defect and central pair agenesis. (F) absent inner and outer dynein arms [37]

The genetics of PCD is complex; with over 30 genes now identified. Most of the genes encode for proteins in the ciliary axoneme however rarer causes of PCD, where no axoneme ultrastructure defect has been identified are also now being classified genetically. Frequently the genetic cause of the disorder is unknown [38] [39] [40].

1.2.1.2 Clinical features of PCD

Symptoms and diagnosis

PCD manifests in organs in which ciliary motility is a critical component of normal function. It is characterised by chronic lung disease, rhinosinusitis, hearing impairment, sub-fertility and organ laterality defects; usually situs inversus (around 50% of cases). Kartagener's syndrome describes the combination of dextrocardia, bronchiectasis and sinusitis, with dextrocardia reflecting abnormal function of the nodal cilia. Additional features are listed Table 1.2-1[41].

Neonatal	
Respiratory	Unexplained respiratory distress Neonatal chest infection
Cardiovascular	Situs inversus
	Cardiac defects
Congenital anomalies	Hydrocephalus
	Oesophageal atresia
	Biliary atresia
Childhood	
Respiratory	Chronic 'wet' cough
	Bronchiectasis
	Atypical asthma failing to respond to treatment
Cardiovascular	Situs inversus
ENT	Hearing impairment due to chronic otitis media
	Rhinosinusitis
Central nervous system	Learning difficulties
Adulthood	
Respiratory	Bronchiectasis
Cardiovascular	Situs inversus
Genitourinary	Male infertility due to impaired sperm motility
	Female ectopic pregnancy

Table 1.2-1: Clinical features of PCD

Patients usually present in the neonatal period with continuous rhinorrhoea, respiratory distress or radiographic evidence of dextrocardia; or in childhood with a chronic productive cough, daily rhinosinusitis or/and otitis media with effusion [42]. Infrequently a diagnosis is made in adulthood following either similar symptoms, investigations for infertility, or following the diagnosis of a family member.

Combinations of tests are used to diagnose PCD. Nasal NO (nNO) is usually low in patients with

PCD (although the cause is unknown) and is used as sensitive screening tool; although occasionally nNO values are low in other conditions therefore it is not used as an isolated diagnostic test [43]. Most frequently the ciliary ultrastructure is examined by transmission electron microscopy (TEM) on strips of nasal epithelium obtained by brush biopsy, allowing defects to be identified. If secondary cilia damage is suspected, ciliated cells can be cultured and re-examined, as secondary damage is absent following ciliogenesis in culture [44]. TEM however does not provide a definitive diagnosis, as cases of PCD with normal TEM are well described and there is currently no 'gold standard' [45]. There is ongoing research on new diagnostic techniques including immunofluorescence labeling and electron microscopy tomography, in addition to genetic mutation analysis [46].

Inflammation in PCD

Sputum from subjects with PCD is predominantly neutrophilic (median neutrophil differential of 70%) [47]. Studies have demonstrated comparatively elevated numbers of inflammatory mediators (neutrophils, IL-8 and protease concentrations) in PCD sputum and BAL compared with subjects with CF of comparable disease severity, both at baseline and during pulmonary exacerbations, in addition to comparatively increased IL-8 in the upper airway [48, 49]. Further research is required to explore the inflammatory profile of the airways in subjects with PCD both at health and during infective exacerbations to better understand how it correlates with disease severity.

Management options in PCD

There are no therapies available that correct ciliary dysfunction, although these are being explored. Currently treatment focuses on managing the downstream effects of the disease (Table 1.2-2).

System	Management
Respiratory	Aggressive use of antibiotics in respiratory exacerbations
	Twice daily physiotherapy and exercise
	Inhaled bronchodilators±corticosteroids
	Regular review with formal lung spirometry
	Yearly review by specialist center
	Annual flu vaccination
	Pneumococcal vaccination
Hearing	Regular audiometry
	Temporary hearing aids may be required for severe hearing loss
Fertility	May require assessment at assisted conception unit
Psychosocial	Genetic counselling
	Primary ciliary dyskinesia support group
	Entitlement to DSS benefits
	Educational statementing due to learning difficulties

Table 1.2-2: List of management options available in PCD [50-53].

The outcome for patients with PCD is diverse. Most patients live an active life with a normal life span, however a minority of patients suffer severe disease, requiring lung transplant, or die prematurely due to respiratory failure or cardiac abnormalities [54, 55].

1.2.1.3 Acquired ciliary defects

Ciliary defects may also be acquired secondary to infection, inflammation and pollutants, which are localised to the area of insult and are reversible following removal from exposure to the toxin [56]. It is essential that these acquired defects are distinguished from PCD.

1.2.2 Comparison of PCD and CF

PCD and CF have many similar characteristics: defective MCC, airway neutrophilic inflammation, chronic respiratory infections and deteriorating lung function. They are often clinically managed in similar ways, with many of the PCD guidelines being extrapolated from evidence from CF, the more prevalent and better-studied disease. There is growing evidence that they should be treated as separate entities due to differences in their clinical expression; different radiological distribution of disease, inflammatory profile and disease progression [47, 57-59]. It is likely that many factors contribute to these differences in disease expression, including the different origin for impaired

MCC; a primary defect in PCD, but normal in CF until it is impaired by secondary processes (discussed below) [60]. Whilst MCC contributes to lung disease in CF, the role of CFTR in immune function is increasingly thought to play a pivotal role in the pathogenesis and account for the faster progressing lung disease seen in CF. Comparison of patients with PCD and CF provides a useful model to further our understanding of the role of CFTR in disease pathogenesis in CSLD.

1.2.3 Cystic Fibrosis

CF is the most common lethal autosomal recessive genetic disease in Caucasians, with an incidence of 1 in 2000-2500 live births [61].The genetic mutation results in a defect in the CFTR protein, which predominantly functions as a cyclic adenosine monophosphate (cAMP) regulated chloride ion channel. CFTR is expressed in the apical surface of epithelia and CF is characterised by the accumulation of viscous mucus within the exocrine organs, which results in obstruction, infection, inflammation and leads to organ failure. Although CF is a multi-organ disease, it is the pulmonary manifestations that are the principal cause of morbidity, and responsible for 90% of the mortality [62]

1.2.3.1 CFTR

The gene encoding CFTR was discovered in 1989 on chromosome 7q31 [63] [64]. Over 2000 mutations in the CFTR gene have been described, however to date less than 150 have been shown to cause CF [65]. Most mutations are rare, with the exception of the loss of the phenylalanine residue at position 508 of the CFTR protein sequence (F508del). Over 70% of patients are F508del homozygous and 90% are heterozygous, although the frequency varies geographically [66]. CFTR mutations are divided into groups according to the stage of the processing of the CFTR protein that the defect arises as illustrated in Figure 1.2-2. Many mutations are as yet unclassified as they are extremely rare; a matter that is being address in the CFTR2 project.

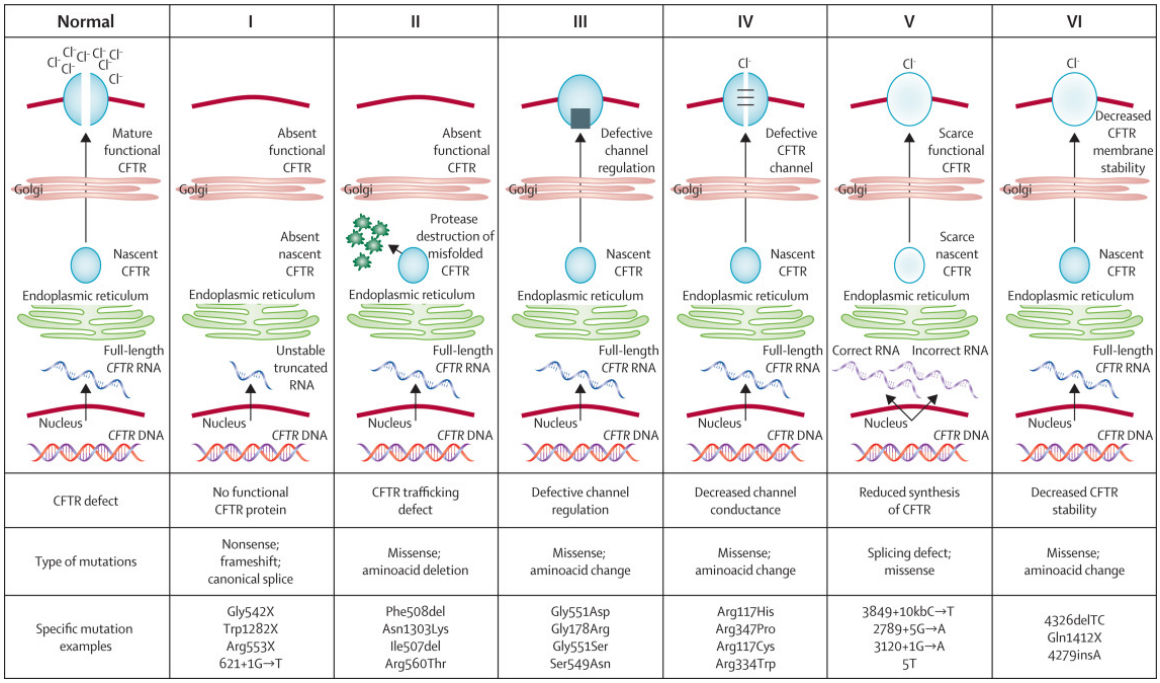


Figure 1.2-2: Summary diagram showing classes of CFTR mutations with examples of disease-causing mutations, Reproduced from Boyle and De Boeck, 2013 [67]

1.2.3.2 Structure and function of CFTR

The *CFTR* gene is translated into an amino acid chain in the endoplasmic reticulum, where it is processed and folded. Quality control is tightly regulated and misfolded CFTR is degraded. A small percentage of normal CFTR moves to the Golgi apparatus for further maturation before trafficking to the cell membrane where the final stage of CFTR processing occurs [68, 69]. It is a glycoprotein comprised of 1480 amino acids that is bound to the cell membrane and is constructed of 2 membrane-spanning domains, which form an ion channel, 2 nucleotide binding domains (NBD 1 and 2) which bind to ATP, and a regulatory domain (R) which regulates CFTR channel opening (Figure 1.2-3).

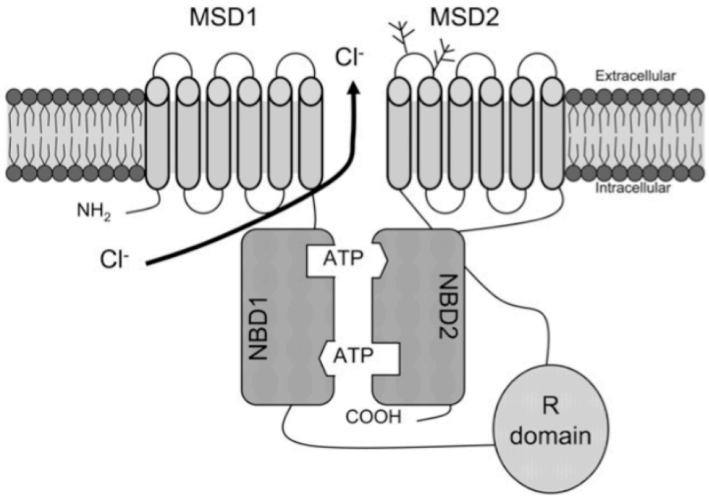


Figure 1.2-3: Proposed structure of the CFTR protein demonstrating its function as a chloride ion channel. MSD- membrane spanning domain; NBD- nucleotide-binding domain; R (regulatory) domain; Cl^- represents a chloride ion. Reproduced from Rogan et al [70]

CFTR is part of a multi-protein assembly in the apical plasma membrane and lies close to a number of other membrane receptors and ion channels. In addition to facilitating chloride ion transport, it is proposed to have multiple other functions listed in Table 1.2-3 [71-73]. Importantly CFTR has an inhibitory effect on the neighbouring amiloride-sensitive epithelial Na^+ channel (ENaC), which results in enhanced absorption of sodium ions by airway epithelium. The exact method of the interaction is currently unknown, and is thought to be either a direct interaction via the transmembrane domains, or indirectly via loss of CFTR transport regulatory substances, loss of chloride transport and/or altered genomic expression.

Functions of CFTR protein
Chloride ion channel
Pathway for bicarbonate (HCO_3), glutathione and thiocyanate
Inhibits the epithelial sodium channel (ENaC)
Activates an associated ATP channel
Sensitises potassium channels to sulfonylurea compounds
Inhibits volume-regulated anion channels
Inhibits endogenous calcium-activated chloride currents
Alters the ion composition of intracelleular compartments
Regulates intracellular vesicle transport

Table 1.2-3: Some important postulated functions of the CFTR protein

1.2.3.3 The role of defective CFTR in the disease pathogenesis

The CF phenotype is complex, even within the same genotype there is a wide variability of disease severity, and a broad clinical spectrum over the different organs and tissues involved. The mechanism by which impaired CFTR function leads to the subsequent clinical picture is currently unknown. Possible theories are discussed below, and are not mutually exclusive.

Impaired CFTR function leads to airway surface dehydration - 'Low volume hypothesis'

The 'low volume hypothesis' attributes the initiation of CF airway disease to airway surface dehydration. Studies under standard (static) conditions demonstrated that, in CF, excess sodium and water is absorbed, and decreased chloride secreted in to the airway. This results in dehydration of the airway surface with subsequent collapse of the PCL, and increased concentration of mucins within the mucus layer, which adhere to the airway surface [74]. Mucus stasis leads to impaired MCC, airflow obstruction and infection. Studies designed to mimic the phasic change in calibre of the airway during normal breathing *in vivo* demonstrated that under these conditions CF ion transport was rebalanced and the PCL volume was restored to adequate levels for MCC, however following infection, the PCL height again depleted, suggesting that airway dehydration in CF appears to occur after an insult to the vulnerable airway [75]. CFTR also contributes to mucus hydration, with absent CFTR function, resulting in a reduction in the water content of mucus, making it more viscous and difficult to clear. This hypothesis is illustrated in Figure 1.2-4 [76, 77].

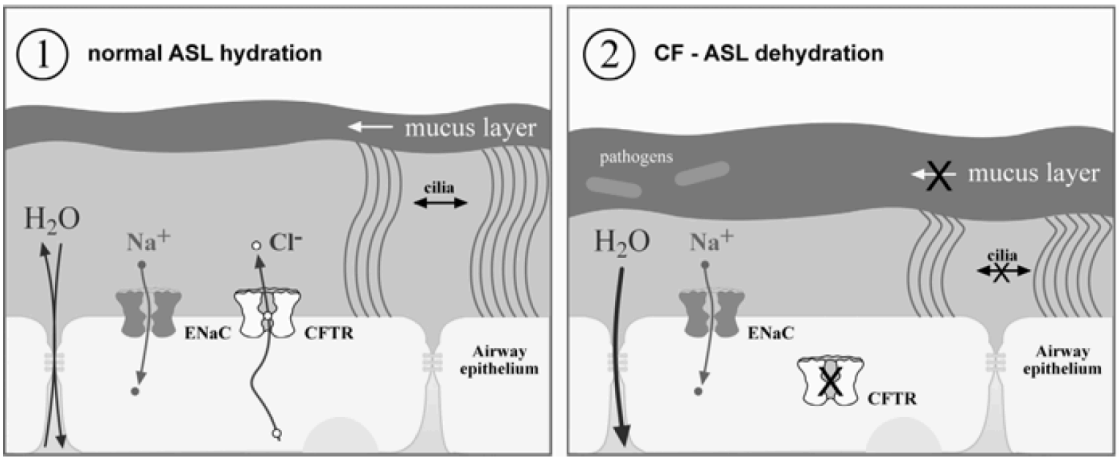


Figure 1.2-4: Illustration of the role of the 'low volume hypothesis' in disease pathogenesis in CF 1) normal ASL hydration in health and 2) the pathological changes occurring in the ASL in the CF airway. Reproduced from Althaus, 2013 [78]

This model has been supported by *in vitro* cell culture systems and animal models demonstrating that decreased ASL precedes impaired MCC, however human clinical studies are difficult to perform, as the volume of ASL is so small, leading to high variability in results due to sampling techniques. [79-82].

Impaired CFTR function leads to alterations in the microenvironment

CFTR in airway epithelia also conducts bicarbonate (HCO_3), which regulates the pH of the ASL [4]. Loss of CFTR impairs HCO_3 secretion and leads to a decrease in ASL pH in preclinical and animal models [83]. CF ASL has been shown to have a lower pH than non-CF tracheal ASL from newborn pigs [84]. In one small clinical study, this difference appeared to diminish with age, with pH in older children and adults with CF comparable to non-CF values. The relevance of this finding to clinical CF disease therefore remains to be determined [85].

Altered ASL pH affects MCC

The composition of ASL in newborn CF pigs has been shown to have an increased viscosity compared to non-CF pigs, thought to be due to the altered pH of its environment, as increasing the pH of the ASL reduced the overall viscosity [86]. These results were consistent with previous groups who had reported that mucins become more viscous at acidic pH [87, 88]. Previous studies had demonstrated that CF mucus in newborn pigs was altered compared to healthy controls, and remained anchored to the epithelial surface compromising MCC [89]. This was shown to persist even when the PCL was adequately hydrated suggesting the mucus abnormality was as a result of a reduced pH in the submucosal glands. The combination of more viscous mucins and adherent mucus are thought to contribute to airway obstruction and impairment of MCC.

Altered ASL pH affects antimicrobial activity

The antimicrobial products in the ASL have individual and synergistic effects designed to rapidly kill bacteria. Studies demonstrated that the reduced pH of ASL in piglets with CF inhibited the antimicrobial activity of these factors. Increasing the pH of ASL with the addition of sodium bicarbonate onto the airways corrected the bacterial-killing defect. This finding directly links the loss of CFTR function to a host-defence defect, which may be a critical step in initiating infection in the newborn lung [14].

Altered ASL pH affects other aspects of the host defence

Some of the other proposed effects of reduced ASL pH on other components of airway defences are listed in Table 1.2-4 [90-94]. It is yet to be established whether normalizing ASL pH e.g. via aerosolised bicarbonate has a therapeutic role in CF lung disease [95]

Proposed effects of reduced ASL pH on the airway defence
Reduced ciliary beat frequency
Regulation of ASL volume
Macrophage phagocyte function
Activation of harmful proteases which are released from over-abundant neutrophils

Table 1.2-4: Proposed effects of reduced ASL pH on the airway defence

Impaired CFTR function in inflammatory cells

Recent evidence suggests that CFTR is expressed in non-epithelial tissues, including immune cells, which may contribute to disease pathogenesis [96]. CF macrophages may have defective ability to destroy pathogens, thought to be due to a pathogen–killing defect. The mechanism by which defective CFTR compromises pathogen-killing is incompletely understood, and it is likely that it may affect a broad range of cellular processes, including inhibiting macrophage activation, and altering pathogen recognition and gene expression leading to altered inflammatory signalling [97]. It was recently shown that both adhesion and chemotaxis were defective in mononuclear cells isolated from patients with CF, although these mechanisms appeared normal in polymorphonuclear (PMN) cells in this study. Another study showed significantly higher levels of cell surface proteins associated with inflammation on PMN cells of subjects with a single Gly551Asp mutation than healthy controls, which normalised following *in vivo* exposure to ivacaftor (discussed below) [98].

It has been proposed that CFTR mutation disrupts phagolysosomal function in neutrophils, plays a role in early neutrophil development, and alters the distribution of surface TLRs thus compromising the innate immune response and dysregulating inflammatory pathways [99-101]. Cells of the adaptive immune response, such as T lymphocytes, may also be affected by intrinsic CFTR dysfunction, which may lead to impaired ability to efficiently clear pathogens [102].

1.2.3.4 CFTR dysfunction and inflammation in CF

The inflammatory response in the lungs of patients with CF has been shown to be self-perpetuating. The neutrophils that are recruited in response to infection synthesise an array of mediators, oxidants and proteases, which are critical for bacterial killing, and are involved in further recruitment of inflammatory cells [103]. Major neutrophil proteases include matrix metalloprotease-8 and -9, neutrophil elastase (NE), and calprotectin, which have all been found in elevated concentrations in the sputum of subjects with CF [104]. This results in a vicious cycle, which eventually overwhelms the host defences Illustrated in Figure 1.2-5. When present in excess, these mediators and enzymes do not enhance bacterial eradication and clearance, but instead, digest structural proteins, weakening the airway structure causing bronchiectasis and contributing to the decline in lung function [105, 106].

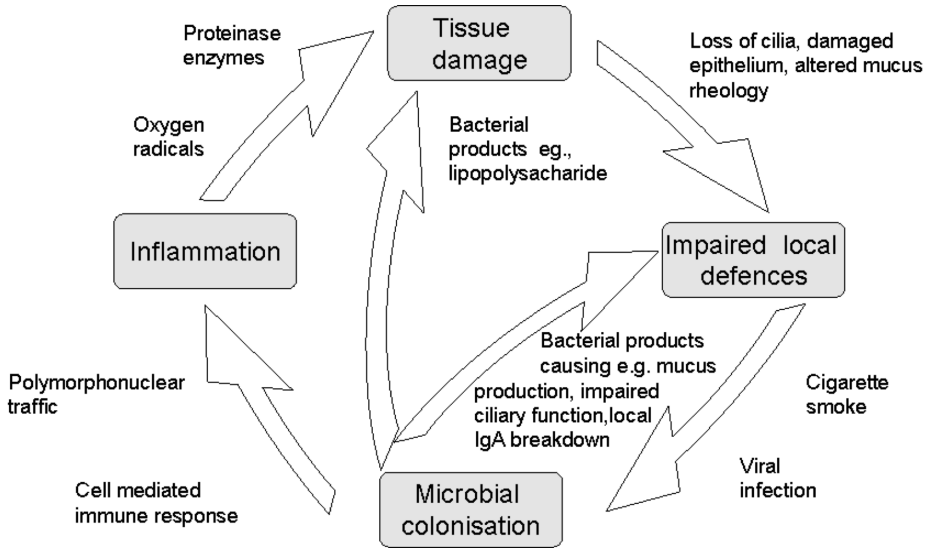


Figure 1.2-5: Vicious cycle of inflammation. Reproduced from Cole et al,1987

There is accumulating evidence that the high levels of inflammatory cytokines and neutrophils in the CF lung may not be a direct response to the accumulation of CF pathogens and bacterial load, but that CF airway epithelia cells have an inherent defect, caused by CFTR dysfunction modifying the airway inflammatory response [105, 107-109]. The evidence supporting the presence of disproportionately high levels inflammation in the CF airway and the proposed mechanisms are covered in Chapter 5.

This section has covered many proposed mechanisms by which defective CFTR is thought to affect the immune system, however this list is not all-inclusive and this is a rapidly expanding field

of research. A better understanding of these processes is required to move forward with our understanding of how CFTR dysfunction results in the clinical features of CF, described below.

1.2.3.5 Clinical features of CF

Diagnosis

The diagnosis of CF requires detection of 2 disease causing genetic mutations, and/or evidence of CFTR dysfunction. Patients are identified either through the newborn screening (NBS) programme or following presentation with characteristic symptoms of the disease. Diagnostic algorithms for different presentations of CF have been developed by the European Diagnostic Working Group [110]. Sweat test is the most clinically utilised method, and measures the concentration of chloride in the sweat, with elevated levels consistent with a diagnosis of CF (>60mmol/L). Following this, CFTR mutation screens are performed to detect disease-causing mutations using the standard, or extended panel if required. If results are inconclusive, CFTR bioassays, which evaluate the ion channel activity are used to look for evidence of CFTR dysfunction, described in 1.3.1.2.

Clinical features

The main clinical features are summarised in Figure 1.2-6

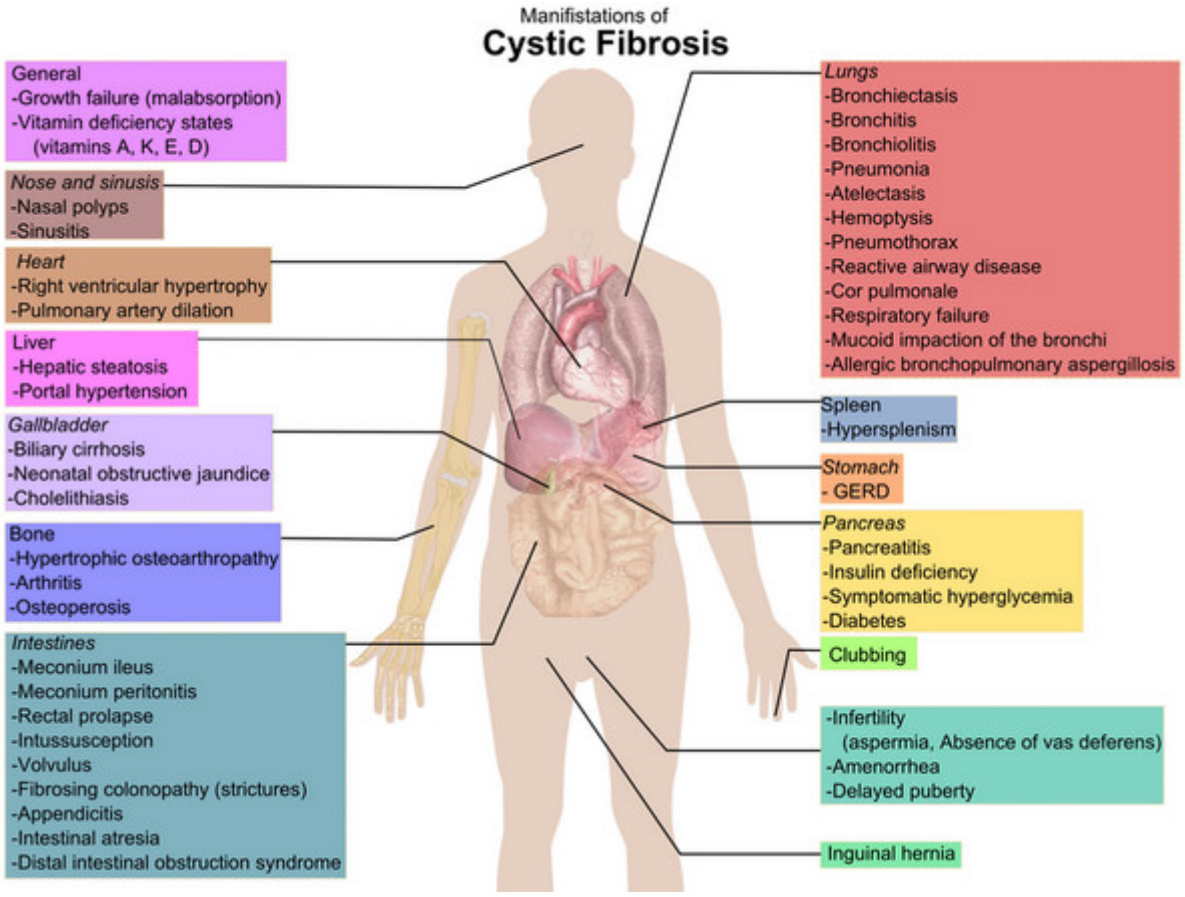


Figure 1.2-6: Summary of the manifestations of cystic fibrosis [111]

Pulmonary

Chronic airway infection leads to inflammation, bronchiectasis and eventually respiratory failure. The lungs of infants with CF are thought to be normal at birth, but become damaged in early childhood [112-114]. Patients characteristically suffer from repeat bacterial infections with common organisms such as *Haemophilus influenza (HI), Staphylococcus aureus (SA)* and *Pseudomonas aeruginosa (PA). PA* is the most predominant airway infection in CF, with studies showing that over 80% of patients are chronically infected with *PA* by the age of 18 [115]. Recent studies exploring the changes in the airway microbiome in patients with CF changes over time using molecular rather than culture based detection methods, reveal it to be more complex than previously thought [116] and groups of emerging CF organisms have been identified [117], in particular anaerobes [118, 119]. The contribution of the extended CF microbiome to airway disease is currently being explored [120].

Pulmonary exacerbations

There is no consensus definition of a pulmonary exacerbation however it is clinically defined by an episode combining some or all of cough, increased sputum production, wheeze, breathlessness, a fall from baseline lung function and elevated markers of inflammation in the blood [121]. The frequency of exacerbations correlates with the severity of lung disease, as patients do not fully regain baseline lung function resulting in progressive decline [122, 123].

Severity of disease

The severity of CF lung disease clinically is measured most commonly using spirometry, and computerized tomography (CT) imaging (described in 1.3.2.1). Spirometry is used longitudinally to monitor disease as it has been shown to correlate with disease severity, morbidity and mortality, and acutely to guide management [124].

Extra pulmonary

15% of infants with CF are born with meconium ileus, caused by inspissated material in the small and large bowels. 85-95% of patients develop pancreatic exocrine insufficiency (PI) and require life long enzyme and vitamin replacements [125]. Other common complications are as illustrated in Figure 1.2-6

Disease progression

When CF was first described (1938) the life expectancy was short. Following the introduction of NBS, the delivering care at specialist centres (MW 103) where patients are treated by a multi-disciplinary team, and developments in treatments, the prognosis has significantly improved [126]. However, patients born today with a diagnosis of CF are only expected to live until an average age of mid 40s if female and mid 50s if male without lung transplantation [127].

1.2.3.6 CF Therapies

Current respiratory treatment options:

Treatment strategies in CF currently manage the downstream effects of the disease to preserve lung function. Antibiotics, delivered either via nebulizer or dry powder inhaler (directly onto the airway) or administered orally or intravenously, form the mainstay of respiratory treatment. In

addition, antibiotics are used long-term to suppress bacterial load and as prophylaxis [128, 129]. Airway mucus clearance therapies (mucolyics such as Dornase alpha, hypertonic saline and mannitol), used in conjunction with physiotherapy, generally result in a modest 3-5% improvement in lung function upon initiation, and a reduction in the frequency of pulmonary exacerbations [130].

Anti-inflammatory therapies have been studied in CF, because the exaggerated inflammatory response is tissue-damaging. Oral corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) had beneficial effects on lung function however the adverse effects are unacceptably high [131]. Azithromycin is the only therapy currently used routinely, which is thought in part to act as an anti-inflammatory agent however the mechanism of action is unknown [132]. Many of the components of the immune response are beneficial and there is a need to preserve these protective aspects. A trial in CF of a leukotriene B_4 (LTB_4) receptor antagonist led to increased pulmonary exacerbations in the treatment group, highlighting the balance of pro and anti-inflammatory mediators in maintaining tissue homeostasis [133].

Nutrition:

Maintaining adequate nutrition with pancreatic enzymes, fat-soluble vitamins and a good calorie intake is essential, as optimal BMI has been associated with less severe disease. Impaired glycaemic control accelerates deterioration in lung function, and improved glycaemic control has been shown to increase long-term survival and lung health [134-136].

Other disease manifestations are managed symptomatically as required. Genetic counseling and psychological support is essential, as is appropriate preparation for lung transplant or palliative management of end stage CF lung disease when required.

1.2.3.7 Novel therapies

Developments in the understanding of the genetics and molecular mechanisms of CF have led to new therapeutic targets, which aim to correct the basic defect itself by either modulating CFTR with small molecules, or by introducing a functional copy of the *CFTR* gene into the epithelial cells using gene therapy. This is a rapidly evolving area of research, which has progressed since this thesis was commenced in 2012. This section describes CFTR modulators whilst gene therapy, a major focus of this thesis is discussed in Chapter 3.

CFTR modulators

These drugs are broadly divided into 3 types; read through drugs, correctors and potentiators.

Read through drugs

These target Class I mutations, which contain a premature termination codon (PTC), aiming to allow the ribosome to 'skip' the premature stop codon (but not the physiological stop signals) leading to a full-length stable and functional protein. Initial trials of 'read through' drugs focused on the therapeutic effects of aminoglycosides however results were mixed, and due to the potential side effects alternative agents were sought [137, 138]. Ataluren (PTC124) is the most promising agent to date, however, despite promising phase II results, the first phase III trial did not demonstrate any clinical efficacy and trials are on-going (ClinicalTrials.gov ID NCT02107859) [139].

Correctors

Correctors aim to address the mis-folding steps that occur in the production of CFTR in class II mutations, to produce a structurally normal protein that can traffic to the apical cell surface. The most studied corrector, lumacaftor (VX-809), improved chloride function in F508del cells *in vitro*, however *in vivo* resulted in only a small difference in sweat chloride with no clinical improvement identified [140]. The combination of lumacaftor with ivacaftor has been assessed in 2 large phase III trials, [141, 142] demonstrating a small but significant improvement in FEV_1 (2.8-3.3% predicted compared to placebo) and a reduction in pulmonary exacerbations, leading to both FDA and EMA approval of a single-pill combination of lumacaftor/ivacaftor (Orkambi) for patients who are F508del homozygous, aged >12 years in July 2015, although NICE have currently not given approval. It does not benefit patients with a single F508del mutation. Alternative correctors are being developed such as VX-661, which are thought to be more effective and following successful phase II trials, and patients are being recruited into phase III trials [143].

Potentiators

Potentiators target CFTR present on the cell surface, increasing the channel open probability. The initial target was 'gating' mutations (class III) in which correctly-localised CFTR protein

demonstrates significantly reduced time open in response to intracellular signals, reducing trans-epithelial ion transport. The most successful potentiator to date is VX-770, (ivacaftor), which was the first therapeutic registered as a CFTR modulator. Ivacaftor increases the probability of opening the CFTR protein via a non-PKA-dependent pathway, both in WT and many CFTR mutations. Trials focused initially on subjects with at least one Gly551Asp mutation as it is the most common class III mutation (approximately 4% of the CF population in Europe) [144]. The exact mechanism by which ivacaftor improves channel opening is unclear, but is thought to be secondary to ivacaftor stabilising the post hydrolytic open state of CFTR [145].

The first clinical trial in 2010, which was a phase II randomised control trial (RCT), established the safety of orally administered ivacaftor BD [146]. Secondary outcome measures demonstrated restoration of CFTR chloride transport with sweat chloride decreasing (towards normal) by (median) -59.5 mmol (range -66 to -19), and a modest increase in chloride transport measured by NPD of (median) -3.5mV (range -8.3 to 0.5). In the phase III RCT, subjects with CF, carrying at least one copy of the mutation Gly551Asp, aged >12, and FEV_1 between 40% and 90% were randomised to receive 150mg BD of Ivacaftor (n=84) or placebo (n=84) [147]. Active patients had improvements in absolute FEV_1 of 10.4% compared to a decline of 0.2% in the placebo group ($p<0.001$) (Figure 1.2-7 a), which were sustained through the 48 weeks of the trial. Secondary outcomes were also overwhelmingly positive: demonstrating decreased pulmonary exacerbations ($p=0.001$), improved quality of life ($p<0.001$) and improved weight ($p<0.001$) in active patients compared to placebo. Sweat chloride measurements decreased, with an average reduction in sweat chloride level to below the threshold for the diagnosis of CF into the equivocal range (Figure 1.2-7 b).

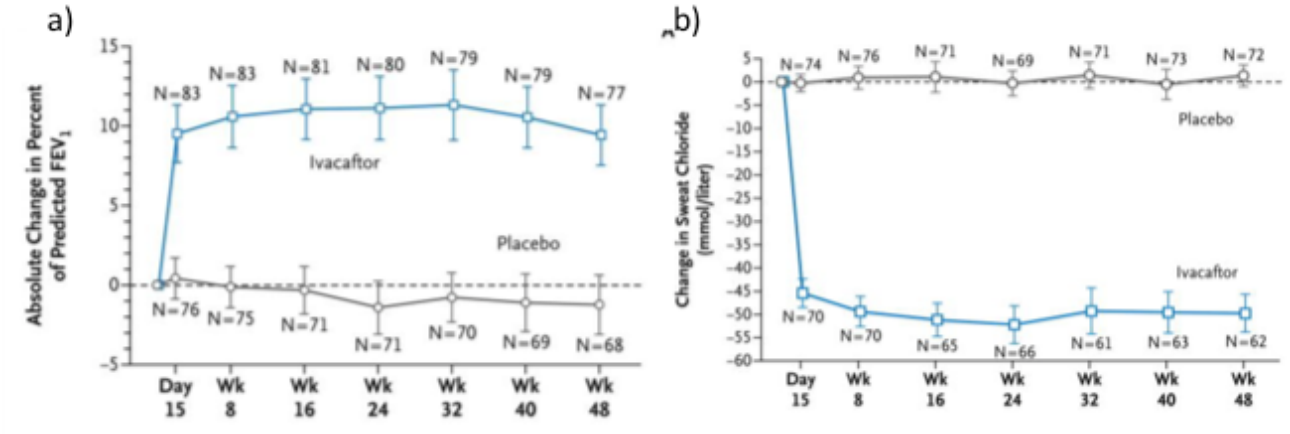


Figure 1.2-7: Changes in outcome measures following treatment with ivacaftor a) the absolute mean change from baseline in percent of predicted FEV_1, through to week 48 b) the mean change from baseline in the concentration of sweat chloride. The values and 95% confidence intervals (indicated by I bars) in both panels are unadjusted. The first data points in both panels are baseline data. Reproduced from Ramsey et al 2011 [147]

These results were replicated in the 6-11 years age group and further trials (PERSIST) demonstrated that safety and efficacy were sustained longer-term, over 144 weeks [148]. In January 2012 the US FDA approved the registration of ivacaftor for patients with CF, aged > 6 years carrying at least one copy of the Gly551Asp gene mutation and the European Commission approved the drug in 2012. Regulatory approval has since been extended to children aged 2-5 years of age after trials demonstrated its' safety in this age group, although reimbursement has not yet been approved in the UK [149].

The use of ivacaftor beyond patients with Gly551Asp has been evaluated with the KONNECTION trial leading to FDA approval for patients with a wider range of mutations, including class IV [150, 151]. Other agents are under development by different companies including Galapagos, who have performed a phase I trial with a CFTR potentiator (GLPG1837) and Concert Pharmaceuticals who have commenced a phase I trial of deuterated ivacaftor [152] [153]

Relationship between restoration of CFTR function and outcome measures of airway disease

Attempts have been made to correlate the restoration of CFTR function with ivacaftor with markers of airway disease to help guide the development of therapies aiming to improve airway disease by specifically targeting the relevant CFTR function. Although ivacaftor led to a dose dependent change in sweat chloride concentration, the decrease was shown not to correlate with improvement in FEV_1 or with improvement in LCI, although the latter has only been explored in patients with mild lung disease [154-156].

The impact of ivacaftor on clinically relevant markers in CF subjects, including respiratory pathogens and airway inflammation, has been assessed in the GOAL study, a longitudinal cohort study of 151 patients eligible to commence ivacaftor [157]. Sputum samples were analysed for NE activity, α1-antitrypsin, secretory leukoprotease inhibitor, IL-1β, IL-6, and IL-8, revealing no significant change over 6 months. There was a significant reduction in sputum culture positivity for *PA,* but not other common CF pathogens, which was replicated when subjects were followed up at 2 years. This may be due to direct interaction between CFTR and *PA* or due to downstream effects of improved MCC. The reduction in *PA* was not associated with change in FEV_1, BMI or hospitalisations, however long-term follow up is being performed [158]. Muciliary and cough clearance were assessed using γ scintigraphy in a subset of patients, which demonstrated

substantially improved MCC. In a small study, ivacaftor was shown to result in a reduction in CRP, however other serum markers of systemic inflammation were not assessed [159].

Studies observing the effect of ivacaftor on innate immunity demonstrated that Gly551Asp mutation had a significantly higher expression of pro-inflammatory surface proteins on neutrophils and monocytes, which was normalized both by *in vivo* ivacaftor treatment and *ex vivo* exposure of blood cells to ivacaftor [98]. In addition, treatment of Gly551Asp patients with ivacaftor has been shown to normalise neutrophil cytosolic sodium, chloride and magnesium ion levels, thus improving degranulation and bacterial killing [160]. These findings suggest CFTR modulators modify host defence cells, which may contribute to the change in clinical picture demonstrated in patients on treatment.

These results are interesting as they enhance our understanding of the relationship between CFTR function, and restoration of ion channel function and elements of airway disease, although longer-term follow-up is required. The lack of a direct correlation between CFTR markers and disease outcome measures suggest either that the outcome measures used are too insensitive, or that the relationship between chloride ion transport and disease status is complex.

1.2.4 Acquired CFTR dysfunction

There has been emerging evidence that external mediators may have an affect on trans-epithelial ion transport, causing 'acquired' CFTR dysfunction. This is a relatively unexplored field, with only a few studies to date exploring this theory, which are reviewed below.

1.2.4.1 Inflammatory mediators

The first of these studies looked at the effect of IFN-γ and TNF-α on sodium and chloride ion channel function in CF and non-CF human bronchial epithelial cells (HBECs) using an Ussing Chamber [161]. The results demonstrated that in both cell types IFN-γ decreased CFTR-related ion transport, and surprisingly down regulated ENaC and up regulated Ca^{2+} dependent Cl^-, thought to suggest that it has a specific modulatory effect rather than causing generalised ion transport defects. This indicates that the up regulation of ENaC in CF may be influenced by factors other than its interaction with CFTR. The effect of ion channel dysfunction following IFN-γ *in vivo* on ASL volume and subsequently the effects on MCC are currently unknown. Similar results were not demonstrated following the application of TNF-α, which the authors hypothesised may be due to

the different signaling pathways involved.

Neutrophil elastase (NE) is a serine proteinase, released by neutrophils, which can contribute to proteolytic lung damage when present in excess, and has been shown to degrade cell-surface molecules including ENaC [162]. *In vivo* studies, measuring CFTR function using nasal potential difference measurements (NPD) in murine epithelium following 7 days of exposure to NE compared to controls revealed reduced chloride secretion, reflecting CFTR inhibition in those treated with NE. These results were repeatable in the presence of *PA,* and were dependent on NE, as CFTR degradation was spared in mice that were unable to produce NE [162]. A possible mechanism for the role of NE in affecting ion-transport is via its interaction with the SPLUNC1 pathway [163]. SPLUNC1 is an extracellular protein that is present in the ASL and is thought to modulate ENaC activity by protecting it from proteolytic cleavage, in order to regulate airway hydration and mucus clearance. Studies have indicated that in health inflammatory mediators, in particular IL-8, upregulate SPLUNC1 secretion, subsequently decreasing ENaC activity and enhancing mucus clearance [164]. Studies indicate that NE can cleave the inhibitory domain of SPLUNC1 from the parent molecule, thus increasing its activity during inflammation [165].

1.2.4.2 Infective microbes

Other studies have explored the interaction between CFTR and the infective microbes themselves, focusing on *PA* as it is the most abundant pathogen in patients with CF. *In vitro* studies demonstrated that non-CF and CF cells exposed to *PA* had reduced chloride secretion, thought to be due to inhibition of endocytic recycling of CFTR due to a factor secreted by *PA*, termed CFTR inhibitory factor (cif) that is capable of reducing apical membrane expression. Cif has been shown to be an epoxide hydrolase enzyme, which is released by *PA* in the airways into outer membrane vesicles and directly secreted into the ASL. When it enters the host epithelial cell, it is thought to diffuse to the cytoplasm and interact with the proteins involved in the final stages of the CFTR recycling process and instead of the recycled CFTR protein reaching the apical cell membrane it is instead degraded by the lysosome, thus decreasing cell surface expression. The resultant loss of chloride secretion and increased sodium absorption result in a reduction in ASL height and ciliostasis (1.2.3.3), and establish an environment in which *PA* infection becomes chronic [166].

1.2.4.3 Other Respiratory diseases

Currently, most work exploring acquired CFTR dysfunction in other respiratory diseases has focused on chronic obstructive pulmonary disease (COPD) due to its high prevalence, and its

many shared characteristic features with CF, including: reduced MCC, chronic neutrophilic airway inflammation and chronic bacterial infections. The epithelial effects of cigarette smoke (CS) contribute to the lung pathology in COPD, although other pollutants and infective agents are known to both initiate and contribute to disease progression; the full mechanisms of pathogenesis remain incompletely understood [167]. Studies have been designed to evaluate the effect of CS on CFTR function in patients with bronchitis and COPD.

In vitro studies have consistently shown an effect of CS on CFTR. The results from a range of studies have demonstrated decreased levels of CFTR protein and reduced protein function thought to be caused by CS inducing rapid internalisation of the CFTR protein, resulting in decreased ASL height, which affects MCC and mucus secretion [168] [169]. The reduction in ASL height was reversed on addition of hypertonic saline.

Experiments performed *in vivo* using NPD evaluated the level of CFTR function in smokers, patients with COPD and healthy controls demonstrating reduced chloride secretion in the nasal epithelium of non-CF subjects who smoked, when compared to healthy non-smokers [168]. Further studies have shown a large, acute, CS induced inhibition of chloride secretion following exposure of CS to the nasal epithelium of healthy ex-smokers who exhibited previously normal CFTR function [169]. A larger study showed that smokers either with, or without clinical symptoms of COPD had reduced CFTR activity measured by NPD compared to age-matched non-smokers, and the reduction in CFTR activity was associated with symptoms of chronic bronchitis [170]. A single study determined CFTR function in the lower airways, using lower airway potential difference (LAPD) measurements in healthy non-smokers, current smokers and former smokers with COPD. The results suggested reduced lower airway CFTR activity in smokers with and without COPD compared to healthy non-smokers [171]. Furthermore, recent data have demonstrated that CS reduces CFTR function at extra-pulmonary sites; with smokers and subjects with COPD having elevated sweat chloride values compared to controls, and decreased CFTR function in intestinal current measurements. This suggests that CS may cause systemic CFTR dysfunction. It is thought that acrolein, a bioactive component of cigarette smoke, may be a causative agent, as *in vitro* studies performed by the same group showed it to be a potent CFTR inhibitor; however it is possible that other agents present in CS may contribute [172]. A better understanding of acrolein and the mechanism of CS mediated CFTR dysfunction may reveal new therapeutic targets for subjects with COPD to alleviate both respiratory and systemic symptoms.

1.2.4.4 Summary

Collectively, these results suggest that external factors may cause an acquired dysfunction in

CFTR. If shown to be the case, this would have important implications. In the subset of CF patients who demonstrate residual chloride secreting capacity, the inflammatory markers may inhibit the residual CFTR function, thus eliminating any protective mechanisms that this may provide [173]. Novel treatments in CF aiming to restore CFTR function may have reduced success in the presence of chronic inflammation and/ or *PA* infection. Beyond CF, in other respiratory conditions in which there is a similar inflammatory profile, such as other CSLD, including PCD the presence of abnormal ion transport may contribute to the burden of disease. Therapies designed to restore CFTR function in CF may also be utilized in a wider population of patients. This has been explored *in vitro* but has not yet been studied *in vivo* to determine the potential benefit in patients with other respiratory diseases [170].

1.3 Outcome measures

Outcome measures are required to assess the effects of defective CFTR on airway health. These must differentiate patients from healthy controls, be easy and safe to make over a range of disease severity, correlate with existing outcome measures and change with the clinical status of the patient. The following sections provide an overview of methods to measure CFTR and disease severity, and their uses and limitations. Finally there will be a review of previous attempts to correlate the measures of CFTR function with disease severity.

1.3.1 Measurement of CFTR

CFTR biomarkers either detect and quantify the amount of protein in the epithelial tissue, or measure its function as a trans-epithelial ion channel. Measurements of CFTR are used to help establish a diagnosis of CF, as an outcome measure in clinical trials for therapies aiming to restore CFTR function as evidence of efficacy, and as research tools.

1.3.1.1 Quantitative assays of CFTR

These assays are exclusively used as a research tool, and are relevant for interventions that aim to increase the quantity of CFTR protein in the cell membrane. Technologies, including TaqMan® Assays, detect both plasmid vector-derived and endogenous *CFTR*-DNA and mRNA. Vector-DNA in the target area demonstrates successful delivery of the product, whereas mRNA indicates successful transfection. The TaqMan probe is a hydrolysis probe designed to increase the

specificity of quantitative polymerase chain reaction (PCR). The probe attaches to a DNA region amplified by a specific primer, and extends to synthesise a nascent strand. When the probe releases flurophore, it is detected by a quantitative thermal cycler and the fluorescence is directly proportional to the amount of DNA template present in the PCR.

Other possible methods to quantify CFTR expression include immunohistochemical techniques, however their use have been limited due to the low abundance of CFTR protein and the low sensitivity of anti-CFTR antibodies [174]. The presence of mRNA may not correspond with the level of function of the protein, and quantitative assays should be used in conjunction with functional assays in the assessment of CFTR correction in the airways [175].

1.3.1.2 Functional assays of CFTR

CFTR function can be measured from the epithelial tissue in any organ in which it is expressed, either *in vitro* or *in vivo*. *In vitro* methods listed in Table 1.3-1, and *in vivo* methods described in the upcoming section. Extra detail is provided for airway potential difference measurements in the nasal and lower airway epithelium as this is of particular relevance to the thesis.

Tissue	CFTR measurement
Single ion channel or cell	Patch clamp - measures voltage across the cell membrane. Predominantly a research tool
Monolayer/ tissue biopsies	Ussing Chamber - measures net ion transport by measurement of the voltage across the epithelial surface. Used clinically to measure ICM on rectal biopsies
Organoids	Measurement of the change in organoid size following exposure to forskolin (a CFTR stimulator). These are a developing research tool

Table 1.3-1: *In vitro* measurements of CFTR function [176-179]

In vivo measurements of CFTR function:

Sweat test

Sweat tests were developed in 1959 after the observation that patients with CF had salty sweat and suffered from episodes of hyponatraemic dehydration [180]. In health, as sweat is conducted

towards the epithelial surface, sodium and chloride are reabsorbed. In CF, with impaired CFTR function, there is a failure of ion reabsorption in the water impermeable duct, resulting in a higher concentration of both sodium and chloride ions in the sweat [181, 182]. To perform a sweat test, sweat production is stimulated by pilocarpine iontophoresis, collected, and the concentration of chloride in the sample is measured. The chloride level is therefore utilised as a surrogate marker for CFTR dysfunction in the sweat ducts.

Sweat testing is the main diagnostic test for CF, with studies clearly demonstrating the validity of measurements in discriminating between patients with CF and non-CF subjects [183]. A recent study looking at the variability of sweat chloride in CF subjects with Gly551Asp showed the between-subject standard deviation (SD) for sweat chloride was 8.9mmol/L (95% CI 7.4-10.6) and within-subject SD was 8.1mmol/L (95% CI 7.5-8.7) [184]. When standardised procedures are implemented, sweat chloride is a useful biomarker in multicenter trials evaluating CFTR potentiators, and it has been used as a major outcome measure in several large phase III clinical trials [154, 185]. Currently, the quantity of change in sweat chloride required to produce a clinical difference is unknown, and its use as an ongoing surrogate measure of clinical response to CFTR modulators is being explored [186].

Measurement of transepithelial potential difference

CFTR regulates ion movement across the cell wall, promoting chloride secretion, and inhibiting sodium influx via ENaC. This trans-epithelial ion movement creates a potential difference (PD) across the cell wall, which is altered in the presence of CFTR dysfunction. PD measurements are electrophysiological tests designed to measure the PD *in vivo* between airway epithelium and a subcutaneous electrode. Measurements assess both chloride channel function and ENaC function independently, allowing real-time evaluation of CFTR and ENaC activity. Measurements can be taken from the nasal epithelium (nasal potential difference (NPD)), and the lower airway mucosa (LAPD).

Nasal potential difference

A catheter is placed against the nasal epithelium and measures the change in PD in response to the infusion of 4 solutions onto the mucosa:

1. The basal PD following the perfusion of a physiological solution (typically Ringers solution),

2. The change in PD following the perfusion of a solution containing Ringers and amiloride (RA) (which blocks sodium channels *in vivo*). In the subsequent solutions, amiloride is present to maintain ENaC blockade throughout

3. Passive chloride conductance (ie, through open channels; not specific to CFTR) generated by exposing the cell surface to a chloride free solution (zero chloride (ZCl)) in the presence of amiloride (ZClA) thus creating an electrochemical gradient

4. Active chloride conductance in response to chloride free solution with the addition of isoprenaline (ZClA), a potent cAMP agonist; this opens CFTR and is therefore considered a more specific test of CFTR function. In practice, steps 3 and 4 are usually summed (total chloride secretion).

Patients with altered/dysfunctional CFTR function (CF) have a characteristic response to these solutions. The increased sodium absorption is reflected by a hyperpolarized (more negative) basal PD measurement. Following perfusion with amiloride there is a larger change in voltage towards zero, demonstrating the inhibition of ENaC. There is reduced or absent re-polarisation in response to both solutions promoting chloride efflux. Figure 1.3-1 shows a PD trace obtained from a healthy control and a typical patient with CF respectively. Abnormal chloride transport is generally accepted as an NPD total response of less than -5mV to both ZCl solutions [187].

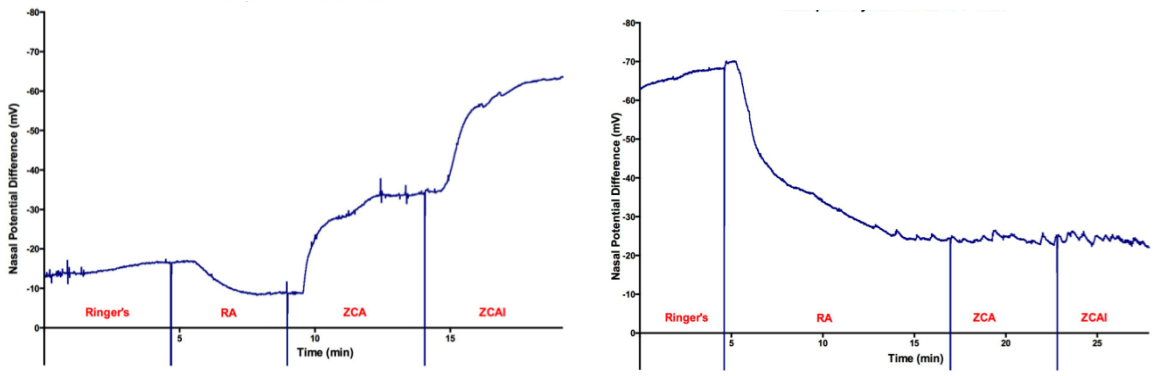


Figure 1.3-1: Examples of nasal potential difference traces from a) a healthy control and b) a CF patient. Phases of the NPD perfusion are labelled; Ringers, Ringers Amiloride (RA), Zero chloride/amiloride (ZClA) and zero chloride amiloride + isoprenaline (ZClA)

Nasal epithelium is commonly used as a surrogate for the lower airway as it is more accessible. NPD has been shown to have strong discrimination validity between CF subjects and controls and

is used to aid the diagnosis of CF in borderline cases [110] [188]. In addition, NPD has been used as a research tool to detect restoration of CFTR function including trials assessing efficacy of CFTR modulators and in gene therapy however currently the minimal change NPD associated with clinical improvement still needs to be determined [137, 146, 189].

Limitations of nasal PD

An experienced operator and specialist equipment are required, the procedure can be time consuming and is technically challenging. There is often significant within subject variability of values, with the coefficient of variation (CV) of basal PD measurements recorded as 13 and 14%, the amiloride phase CV 33%, and even greater in the chloride response [190, 191]. There is inherent physiological variation, with CFTR expression not a static process however PD measurements are also affected by non-specific tissue damage e.g. nasal inflammation (secondary to rhinosinusitis), polyps and scarring. The epithelial damage results in a 'blunted' trace with a lower (less negative) basal value and reduced amiloride and chloride response [192].To minimise operator and inter-centre variability (important for multi-centre trials) standard operating procedures (SOPs) have been developed by the European Cystic Fibrosis Society (ECFS) Diagnostic Network Working Group to help standardise the procedure [193].

Lower airway potential difference

The lower airway manifests the majority of disease, and CFTR function in the bronchial epithelium may be more relevant clinically. LAPD measurements use a modified method to that used in the upper airway [194]. The technique is performed by inserting a single lumen catheter down the channel of a bronchoscope, which is used to both perfuse solutions and record measurements and can be used to

The amiloride phase is omitted as the solution would pool in the lower airways and affect subsequent measurements. Basal measurements are taken in the proximal lower airway (level of the carina) and the basal PD, and the response to perfusion of ZCl/Iso, is measured typically in 3 different areas in the distal airway (approximately 5th generation). Figure 1.3-2 demonstrates a LAPD trace obtained from a control and a patient with CF respectively, with reduced chloride secretion demonstrated in the CF patients.

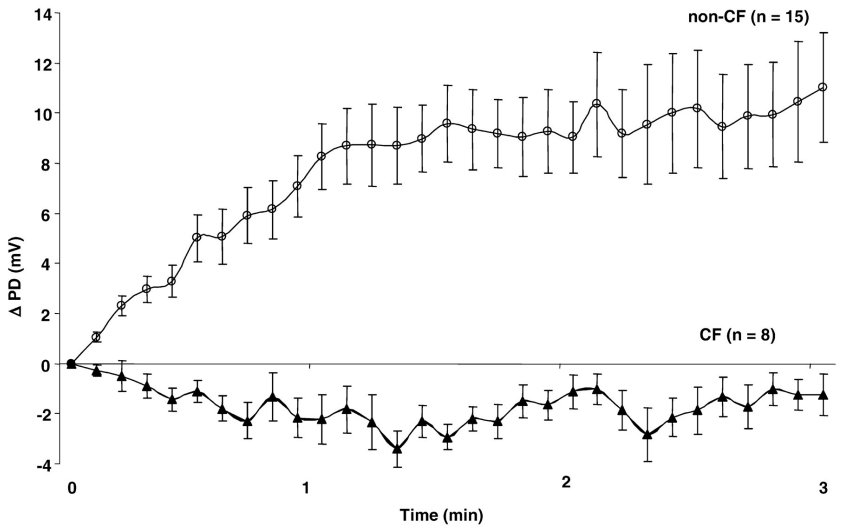


Figure 1.3-2: LAPD measurements in a 3rd generation airway demonstrating chloride secretion in response to a zero chloride (ZCl) solution in CF children and non-CF children during 3 minutes ($p<0.001$). Reproduced from Davies et al 2005 [195]

LAPD has been shown to successfully discriminate between subjects with and without CF including children [195]. As LAPD is more invasive than NPD, it is performed much less frequently and is used entirely in a research setting. It has been developed as an outcome measure for gene therapy trials described in Chapter 3 and 4, and was used in a study to explore CFTR function in COPD (Section 1.2.4.3).

Limitations to Lower airway PD

Measurements require anaesthesia and bronchoscopy and are therefore associated with increased risk and complications. The technical difficulties associated with the procedure set up will be discussed in Section 2.2.8.2. The procedure takes longer to perform than NPD, and the environment (in operating theatres) results in more electrical interference. In addition, lower airway mucus and secretions obstruct the contact between the catheter and the cell surface, interfering with the measurements. There are limited data on the variability of LAPD measurements and how they change in the presence of disease. A major limitation of this technique is the lack of amiloride perfusion stage in this procedure, which decreases the ability to monitor ENaC function, although the baseline PD provides some information.

Currently there is little evidence comparing NPD results with those obtained using LAPD

techniques in the same subjects. This is currently being addressed by Dr Michael Waller in his PhD thesis [196].

1.3.2 Measurements of airway disease and inflammation

There has always been a clinical need to detect both acute and chronic changes in CF lung disease; to allow for appropriate and timely medical interventions, to provide a means to evaluate therapeutic success, including medications acting directly on the CFTR protein, and to monitor disease progression longitudinally. CF outcomes have improved over recent years and as a result, more sensitive biomarkers are required, particularly in the children and for those with mild lung disease.

The downstream effects of CFTR dysfunction include infection, inflammation and subsequent lung damage and methods to quantify these effects have been developed. The most obvious limitation of these downstream measures is that they are not specific to CFTR dysfunction. The following section provides an overview of the most important outcome measures currently used divided into; physiological, structural (radiographic) and measures of inflammation.

1.3.2.1 Physiological outcome measures

Measures derived from spirometry

Forced expiratory volume in the 1st second (FEV_1) is the most commonly used measure derived from spirometry and is currently the 'gold standard' measurement of lung damage in CF and other respiratory diseases; used in both clinical practice and research trials. Spirometry is quick, easy to perform, non-invasive and does not require specialist equipment or operators. Large cross sectional and longitudinal studies have generated 'normal' values allowing function to be evaluated. FEV_1 is thought to mainly reflect pathology in the upper, larger airways, and is insensitive to distal airway disease.

Other measures derived from spirometry are also commonly used to assess lung disease in patients with CF. Forced expiratory flow at low lung volumes, most usually 25-75% of FVC (FEF_{25-75}) are thought be a more sensitive than FEV_1 in the detection of obstructive small airway disease [197]. Spirometry is difficult to perform in young children, as it requires co-operation and the success of the procedure can be variable, particularly in the pre-school age group. [198].

FEV_1 is currently the only primary pulmonary endpoint recommended by the European Medicines Agency for CF clinical trials [199]. However, although it is useful in advanced disease states, it has long been recognised as insensitive to detect early and mild disease with the mean rate of decline measured to be about 2% predicted per year [200]. New outcome measures are required for clinical monitoring and trial outcomes for patients with mild pathology, especially children.

Multiple breath washout tests and derived measures

Multiple breath washout (MBW) has been performed for over 50 years and involves monitoring a tracer gas as it is washed out of the lungs during tidal breathing [201]. The gas can be exogenous and inhaled into the lungs to equilibrium prior to washout commencing, or may be endogenous nitrogen that is normally resident in the lungs, which is washed out following the inhalation of 100% oxygen. With each successive breath of the washout there is a fall in the peak concentration of the tracer gas, Figure 1.3-3.

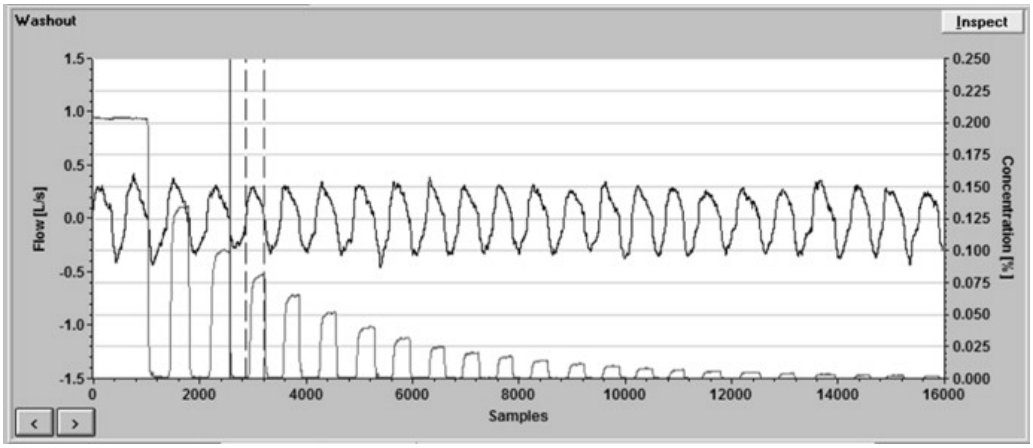


Figure 1.3-3: Typical washout tracing in a healthy subject. Flow is shown in the upper trace with the scale on the y axis. Tracer gas (0.2% SF_6) concentration is shown in the lower trace. Reproduced from Horsley et al 2009.

Lung clearance index

Lung clearance index (LCI) is the most commonly used parameter derived from MBW tests. It is used to detect gas mixing efficiency and is defined as: the number of resting functional residual

capacity (FRC) turnovers (TO) required to wash out the tracer gas, with tidal breathing from equilibrium to a pre-defined end point. LCI is therefore calculated from the total volume of air breathed out known as the cumulative expired volume (CEV) during the washout divided by the FRC.

$$\text{Lung clearance index (LCI)} = \frac{\text{Cumulative Expired Volume (CEV)}}{\text{Functional Residual Capacity (FRC)}}$$

Conventionally, the end point is where the end-tidal concentration reaches $1/40^{th}$ of the initial concentration as this represented the limits of the original N_2 analysers. When there is airway disease resulting from mucus retention, inflammation and patchy obstruction, a greater number of breaths are required to eliminate the gas, resulting in a higher LCI. LCI measures overall ventilation heterogeneity and is thus thought better to reflect airway disease in the small airways better than FEV_1.

MBW requires only passive cooperation and can therefore be achieved in young and unwell subjects. It is a safe and non-invasive outcome measure and is a repeatable and reliable marker of airway damage [202]. The intra-visit repeatability CV is between 3 and 8% [203] [204], with mean inter-visit reproducibility of 0.6u (measurements approximately 10 days apart) in healthy adults [203], and a CV of up to 9% in subjects with CF. LCI is increased in CF patients compared to healthy controls [205] from as early as 10 weeks of age up to adulthood [206], and correlates both with structural lung changes [207] and other markers of inflammation [208]. Most significantly, LCI has been shown to be more sensitive to CF lung disease than standard spirometry (FEV_1), with abnormal results in patients with normal spirometry and lung volumes, providing an opportunity to detect early airway dysfunction in what was previously considered the 'silent years' in pre-school children and in subjects with mild disease [209-212].

Use of LCI in clinical care and research

Recent studies have shown that LCI is dependent on both age and height, principally in the first 5 years of life [205]. It is important that dead space is minimised. A correction factor has been suggested to adjust for dead space although more studies are required to assess whether this is useful [213]. From aged 6 and above LCI is a useful tool for tracking disease progression and is being increasingly used in specialist respiratory centres. No trial of LCI-guided decision-making has yet been conducted however.

Studies exploring the role of LCI in evaluating the effectiveness of short-term interventions have produced inconsistent and variable results, including with chest physiotherapy and iv antibiotics [214] [215-217]. A possible explanation is that interventions that cause movement of mucus may either result in recruitment of additional lung units not previously contributing to the MBW, or blockage of other lung units, leading to variable changes in LCI. LCI should be viewed in combination with standard lung function and interpreted within the clinical setting [218].

LCI has been used as an outcome measure in a small number of clinical trials for mucus clearance agents, demonstrating a significant treatment effect and supporting its use as an outcome measure [219, 220]. More recently, LCI has been used to explore the effect of novel therapies in CF on airway dysfunction [156]. To date, the minimal change in LCI considered to be clinically significant is unknown, but if the measurement is to be useful, it must be larger than the difference seen between repeat measurements without intervention and no change in clinical status.

Limitations of MBW techniques

MBW requires expensive equipment and an experienced operator to perform and analyse the measurements. It is necessary for centres to align methods according to published consensus guidelines as different machines and analysis packages are not interchangeable [221] [222]. Measurements can be time consuming, particularly if performed on patients with severe lung disease. To date, much of the research surrounding LCI has focused on subjects with mild lung disease and relatively preserved lung function and its use in detecting and monitoring disease changes in subjects with a wider range of disease severity is yet to be established.

1.3.2.2 Radiographic outcome measures

Plain x-ray

Plain x-ray is of limited value in the diagnosis of bronchiectasis and has been shown to have a sensitivity of only 37% [223].It is not a viable option for monitoring subtle changes in disease activity, although it can be useful in the event of acute deterioration.

High Resolution Computed Tomography (HRCT) Scans

HRCT imaging of the chest provides structural information and has a sensitivity of 97% for the diagnosis of bronchiectasis [224]. Early manifestation of CF lung disease includes the presence of regional air trapping and increased bronchial wall thickness progressing to bronchiectasis, mucus plugging and progressive lung pathology. Scoring systems have been developed to standardise the interpretation of the images thus increasing their utility as an outcome measurement [225]. HRCT scans are more sensitive than other measures in monitoring disease progression, although this is limited in patients <1 year old, as CT changes during this period may be minimal [226, 227].

The major limitation of HRCT in routine monitoring of patients is the radiation exposure, which may be associated with a radiation-induced risk of cancer [228]. Recent developments have led to protocols in which the dose is reduced by 40-50%, however these are not always available, and significant doses of radiation are still involved [229]. To date no study has demonstrated that regular HRCT improves clinical outcomes in CF.

MRI, which is a radiation-free imaging technique, has been shown to sensitively detect CF lung disease (structure and function), even in pre-school children, and detect changes associated with pulmonary exacerbations. Following recent technical advances it is emerging as a tool for non-invasive monitoring of disease [230].

1.3.2.3 Markers of inflammation

Inflammatory markers are measured both systemically in the blood and more locally in the sputum, obtained via induced or spontaneous expectoration of sputum or in bronchoalveolar lavage (BAL) samples. Novel methods are being are also being developed to analyse the level of inflammatory markers in nasal secretions, exhaled breath condensate (EBC) and urine, which are currently being evaluated as surrogate markers for the airway [231, 232].

Blood

Systemic markers of inflammation include total white cell count (WCC), peripheral blood neutrophils, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). These are used commonly in adult clinical practice, and are elevated during infection and fall with antibiotic treatment in patients with CF [215].

Other markers utilised in research include calprotectin, a marker of neutrophilic inflammation, and the level of pro-inflammatory cytokines [233]. Our multi-centre observational study of adult and paediatric patients with CF (>10 years), the 'tracking study', evaluated the change in conventional and novel biomarkers of CF lung disease at the beginning of a CF pulmonary exacerbation and following a course of iv antibiotic therapy. A significant reduction was seen in serum CRP, IL-6 and calprotectin following treatment, no changes were observed in levels of IL-8 or TNF-α and IL-10 levels were undetectable [215]. There were no changes in levels of sputum markers.

Sputum

Sputum analysis may be a useful tool in the assessment of disease. Clinically, the most commonly assessed markers are purulence, and presence of microbial growth on culture [234, 235]. In research, additional factors include: volume collected over 24 hours, proportion of solids (percent dry weight), DNA content, viscosity, total and differential cell count and the level of pro-inflammatory markers.

Studies correlating sputum biomarkers with disease severity have shown that levels of NE, matrix metalloproteinase (MMP-2 and MMP-9) anti proteases (NE a1 anti protease complexes [NEAPCs], secretory leukoprotease inhibitor [SLPI] and tissue inhibitor of metalloproteinase 1 [TIMP-1]) have the best predictive values for subsequent lung function decline [236], and elevated NE, neutrophil counts and IL-8 had a significant longitudinal association with decreased FEV_1. However others have found no correlation, and there is ongoing controversy regarding the role of these markers in monitoring airway inflammation and disease. Children are often unable to expectorate when required, although sputum production can be induced by using nebulised normal or hypertonic saline [237]. BAL collects fluid which is often of variable quantity and dilution; results may therefore be poorly reproducible. It is an invasive procedure and therefore only performed when clinically necessary.

Bronchial biopsy

Endobronchial biopsies have been shown to detect early pathological changes in the CF lung, demonstrating amongst other changes; increased basement membrane thickness, loss of ciliated epithelial cells and ultra structure abnormalities, goblet cell hyperplasia, increase in airway smooth muscle, and bronchial mucosal inflammation. These are associated with infection and inflammation, and provide insight into the mechanisms of airway remodeling in CF [238]. Limitations include that they only detect changes in the very small of proportion of the airway that

was sampled, and only in the proximal regions. Biopsies are invasive and this procedure cannot be recommended as a routine method of monitoring airway inflammation.

Nasosorption

Novel methods have been developed to collect nasal mucosal lining fluid which utilising a synthetic absorptive matrix (SAM). Strips of the material are passed into the nostril to collect nasal secretions which are then analysed to assess the levels of inflammatory markers. This method, known as nasosorption, has been shown to be superior to previous methods in recovery of cytokines however its use in monitoring disease status in CF has yet to be determined [239, 240]

1.3.3 Relationship between quantity of CFTR and disease

Since the discovery that a defect in CFTR protein leads to CF, studies have been performed to try and correlate the level of CFTR dysfunction with the severity of lung disease measured physiologically, structurally and by levels of infection and inflammation (above), and to try and quantify how much correction may be required to produce a clinical benefit. Better understanding of these issues will guide the development of novel therapies which aim to restore CFTR function; to help determine if a therapy will be effective, to measure its efficacy and to help establish dosing regimens. We know that carriers are to all intents and purposes normal, so 50% of CFTR function is sufficient.

1.3.3.1 CFTR and disease phenotype

Attempts to correlate sweat chloride concentration and clinical phenotypes have shown inconsistent results to date. Initial studies observed lower sweat chloride concentrations among patients with class IV mutations (associated with a milder disease) however within other class mutations, sweat chloride concentration does not correlate with pulmonary status, even in the subgroup of patients who are pancreatic sufficient and thought to have residual CFTR function [173, 241, 242]. As discussed, whilst the values of sweat chloride concentration, and FEV_1 and LCI showed a significant improvement in patients with the *Gly551Asp-CFTR* mutation following treatment with ivacaftor, the changes were not found to correlate.

The relationship between NPD measurements and disease severity CF is also undetermined. Some studies have shown no relationship with respiratory function, whilst others have shown a

correlation with a higher (more negative) baseline PD and a more exaggerated amiloride response (both indices of sodium hyperabsorption) with worse respiratory function and long-term survival and a normal basal PD with milder respiratory disease [243, 244] [173, 187, 245, 246]. A study of adult CF patients (n=79) demonstrated a relationship between increased chloride secretion and pancreatic status but not pulmonary phenotype, and a study of older CF patients failed to show a correlation between survival and residual CFTR chloride function in patients with severe CF mutations [247-250]. In contrast other studies have reported that residual chloride secretion was associated with higher FEV_1 regardless of the genotype, and a statistical correlation has been reported between stimulated chloride transport in male patients with CF and FEV_1, however this was not replicated in a comparable female cohort [249]

A study exploring CFTR dysfunction in bronchiectasis patients with only one mutation in *CFTR* indicated that there is a continuum in airway ion transport which is strongly associated with *CFTR* genotype and clinical phenotype [251].

1.3.3.2 Level of CFTR required to achieve a clinical benefit

In vivo studies suggested that low-levels of correction of CFTR expression in the airway epithelium can restore chloride transport levels to normal, however later studies indicate that nearer 25% may be required to allow efficient mucus transport. Recent animal studies demonstrated that low levels (7%) of adenovirus-mediated *CFTR*-gene expression into the sinus epithelial cells of CF-pigs can produce detectable levels of chloride correction, and that transfer into 20-30% of cells corrected chloride transport to 50% of non-CF levels. However, it is unknown what levels of chloride correction are required to produce a clinical benefit. Different organs require different levels of CFTR function to maintain function. The vas deferens is extremely sensitive to CFTR. Congenital bilateral absence of the vas deferens (CBAVD), which is present in most male CF patients, can also be a single-organ condition leading to male infertility. These men often have mutations in CFTR leading to partial function [252]. It appears that CFTR function of approximately 10% of normal causes disease in the genital tract but is sufficient to prevent lung disease. As discussed, it is unclear which features related to CF pathophysiology are the most important, and the amount of CFTR expression required to correct bicarbonate transport, mucus unfolding and ASL pH are currently unexplored.

1.4 Rationale for studies in this thesis

It is clear that CFTR is vital to maintain airway health; however it is incompletely understood how impaired CFTR function results in the chronic inflammatory lung condition characteristic of CF. A better understanding of this process may help guide the development of novel therapies in CF that aim to restore CFTR function and to identify potential future therapeutic targets. In addition, if secondary CFTR dysfunction exists in other chronic suppurative lung diseases, it may contribute to the disease progression, and treatments that are useful in CF may be beneficial in a wider patient group. This thesis explores the relationship between CFTR ion transport function and markers of disease severity in order to better understand the complex relationship between CFTR, inflammation and disease progression. The start of a Multidose Trial of *CFTR*-gene therapy provided the unique opportunity to explore the effect of the restoration of CFTR by these novel therapies on markers of airway health.

1.5 Hypotheses, aims and objectives:

1.5.1 Hypothesis

1. There is a direct relationship between CFTR function as manifest by sodium and chloride transport and airway health

2. There is a bidirectional relationship between CFTR function as manifest by sodium and chloride transport and airway inflammation, namely dysregulated chloride and sodium transport leads to inflammation and inflammation which in turn further impairs ion transport

1.5.2 Aims

1. To demonstrate the relationship between CFTR functional deficiency as manifest by dysregulated chloride and sodium transport and clinical markers of airway disease

2. To define the relationship between CFTR function as manifest by dysregulated chloride and sodium transport and airway and systemic inflammation

1.5.3 Objectives

1. To measure lower airway potential difference and relating it to functional and structural markers of airway health in patients with CF

2. To establish the relationship between changes in airway PD and those in clinically relevant markers after restoring CFTR function by repeated dosing with *CFTR* gene therapy

3. To compare the inflammatory response to bacterial challenge of CF nasal epithelial cells to those of wild-type and non-CF CSLD, both of which have presumed normal CFTR function

4. To assess CFTR function as manifest by dysregulated chloride and sodium transport using nasal PD in patients with secondary CFTR dysfunction in the CSLD, PCD, and relating it to the severity of inflammation

The next chapters detail the methods I have used to address these research questions. Techniques common to several studies are described in Chapter 2; techniques specific to particular studies are described with the relevant results in subsequent chapters.

2 General methodology

The overarching theme of this thesis; exploring the relationship between loss of CFTR protein ion function and markers of disease severity in chronic suppurative lung disease, was explored in four studies. This chapter describes those parts of the methodology which were common throughout the work of thesis, whilst the methodology specific to a single study are described in the relevant Chapters (3-6). First, there is a definition of the diagnostic criteria used to confirm the subjects' eligibility to participate, which was common throughout, followed by details of the methodology of the outcome measures.

2.1 Disease group definitions (subjects)

Between March 2012 and September 2014 patients were recruited to take part in either one or more of the studies outlined in Chapters 4-6. Subjects were defined as below:

Cystic fibrosis:

CF patients were required to have had the diagnosis confirmed by sweat test (sweat chloride levels > 60 mmol/ml) or by genetic analysis demonstrating 2 mutations recognised to be CF disease causing, in addition to clinical features as in the European Diagnostic Guideline [110].

Primary ciliary dyskinesia:

PCD patients were required to have had the diagnosis confirmed by high-speed video-microscopy, or assessment of ciliary ultrastructure by transmission electron microscopy (TEM) revealing a ciliary defect known to cause PCD, or by genetic analysis demonstrating a mutation known to cause PCD and the presence of sinopulmonary disease.

Healthy controls:

Healthy controls were recruited from hospital staff with no history of respiratory disease, congenital cardiac disease, neuromuscular disease or history of premature delivery. All controls were required to be non-smokers and to be free from bacterial or viral infections of the upper respiratory tract symptoms for the preceding 4 weeks. The demographics of the control group was controlled so as

to not differ significantly from that of the patient population. *CFTR* mutation analysis was carried out on healthy controls to ensure that they were not carriers of a mutation (2.2.7).

2.2 Outcome measures: methods

2.2.1 Baseline measurements (Chapters 3,4,6)

Height was recorded for both adults and children using a Leicester height measure and measured to the nearest centimetre. Weight was measured with shoes and outdoor clothing removed, and documented in kilograms, corrected to the nearest 100g.

2.2.2 Spirometry (Chapters 3,4,6)

Spirometry was performed using an EasyOne Spirometer attached to a disposable, single use EasyOne spirette. Calibration was performed daily using a 3 litre syringe, and was considered successful if the results were +/- 3%.

Subjects attached a nose clip sealing both nostrils prior to starting the procedure. They were asked to breathe in until their lungs were full, take the EasyOne in both hands and seal their lips around the spirette to ensure no leak. They were then instructed to breathe out as hard and fast, with as minimal delay as possible from the inspiration and the breath was continued for as long as possible to ensure complete emptying of the lungs. If successful, the EasyOne instructs the patient to repeat the manoeuvre a minimum of 3 times with at least 30 seconds recovery time in between tests to ensure that the data was good quality. The EasyOne screen displayed a ‘Session Complete! Good Job!’ message if the within-manoeuvre and between-manoeuvre quality fulfilled the European Respiratory Society (ERS)/ American Thoracic Society (ATS) acceptability criteria, and the spirometry results were displayed on the screen [253]. Reasons for classifying a single manoeuvre as unacceptable include beginning too early or too late, if disturbed flow was detected e.g. by a cough or if the manoeuvre was too short. The difference between the FEV_1 and/ or the FVC of the last and previous manoeuvre(s) was required to be <150ml (or 100mls if the FVC was less than 1L). If the session did not fulfil the criteria, the subject was allowed a 10-minute break, and the procedure was repeated a maximum of 3 times to ensure that the subject did not tire. It was very rare that repeats were required.

To enable a single set of reference equations to be used in all ages for spirometry, the data were converted to percent predicted using the reference ranges produced by Stanojevic et al [254]. Data were converted using a plugin available from www.growinglungs.org.

2.2.3 Multiple breath washout tests (Chapters 3,4,5)

MBW was conducted either using the Innocor gas analyser coupled with 0.2% SF_6 tracer gas.

2.2.3.1 Innocor

MBW tests were performed as according to the UKCFGTC 'multiple breath washout using modified innocor device' SOP which was developed using published guidelines [212]. The Innocor (Innovision, Odense, Denmark) is a photoacoustic gas analyser that has been validated to measure LCI in CF [203]. The Inncocor device uses 0.2% SF_6 as the tracer gas as there is a high signal to noise ratio with this analyser throughout the washout.

2.2.3.2 Equipment

- Innocor device
- Supply of 0.2% SF_6 in air (BOC Ltd. Size L tank 2000ppm SF_6 air/200)
- Single use sterile filters:
- Adult – Air Safety Ltd. Bacterial/viral filter 4000/01
- Paediatric – Air Safety Ltd. Bacterial/viral filter 9070/01
- Clean mouthpiece and noseclips
- Mouthpiece (Ferraris)
- 3L calibration syringe
- Electric fan(s)
- Innocor log to record settings

2.2.3.3 Preparing the equipment

Figure 2.2-1 illustrates the set up equipment for the MBW test using the Innocor.

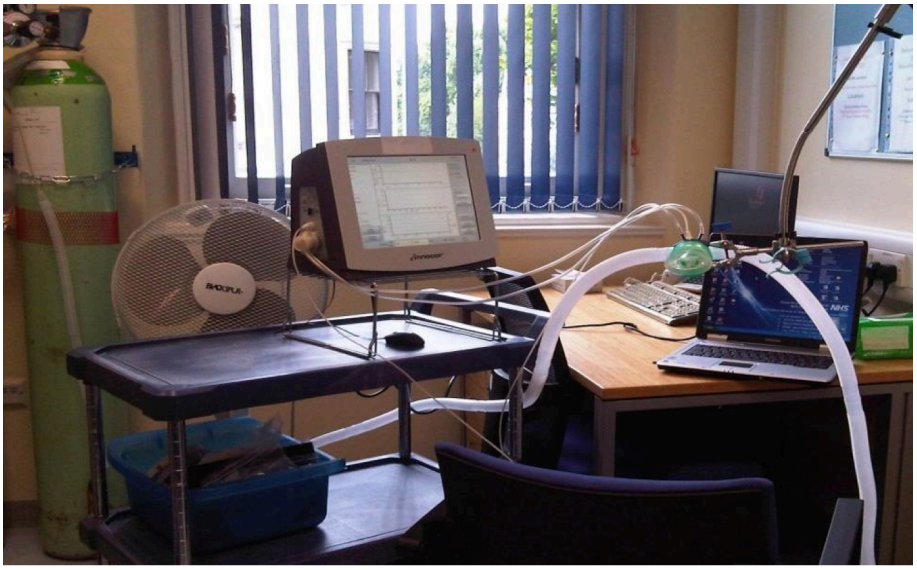


Figure 2.2-1: LCI set up using the Innocor^{TM} with SF_6 as a tracer gas. The photograph shows the patient mouthpiece and filter with the flow pass of SF_6 (left to right). The fan is to free the room of exhaled SF_6 to prevent re-inspiration during the washout

Calibration

Flow-gas delay calibration was performed daily on the Inncocor. The operator breathed out slowly into the mouthpiece to establish a flat plateau of expired CO_2 concentration. A sharp breath was then taken, with inspiration continued for at least 2-3 seconds, to ensure a sudden transition of flow from expired to inspired, leading to the CO_2 concentration falling to ambient levels very quickly and staying there. The mean CO_2 delay value was recorded. Calibration was acceptable if the individual gas delay did not vary by more than 20 ms, and did not vary compared to the previous days value by more than 20-40 ms.

Flowmeter calibration was performed daily on the Innocor using a 3 litre calibration syringe. The syringe was filled and emptied by hand 5 times in total: 2 slow (1/2 - 3/4 L/s); 2 medium (1 1/2 L/s); and 1 fast (2 1/2 L/s). Speed was approximated. The volume measured at all rates was required to differ by less than +/- 2% (between 2.94 and 3.06L) to be acceptable. This was repeated if the initial calibration had been unsuccessful.

2.2.3.4 Performing MBW measurements

The Innocor was turned on for at least 20 minutes before testing to allow the photo-acoustic gas analyser to warm up. Ambient temperature and humidity, flow meter calibration, and flow gas delay were recorded in the Innocor Log.

Patients were asked to sit upright with legs uncrossed as this can affect functional residual capacity (FRC). A nose clip was attached to ensure no air leakage and the patient engaged with the mouthpiece, achieving a good seal. The patient was instructed to breathe 'normal/ relaxed' breaths.

Wash-in Phase

The tracer gas (0.2% SF_6) was switched on at 10-15L/minute. After 1-2 minutes the patient's breathing was generally at a comfortable, steady tidal volume and the Inncocor test screen tidal volume graph could be used to determine the patient's breathing pattern. The Innocor machine reported the maximum, minimum, and the difference between these 2 SF_6 concentrations. When this was 0.003% or less for three successive breaths the 'wash-in' phase was complete.

Disconnection of flow-past

The fan was then turned on and the patient asked to continue breathing normally as the gas was going to be disconnected. The subject's chest movements were observed, and early in the expiration, the T-piece was rapidly and smoothly disconnected and covered with the operator's hand. The tubing was quickly removed so that the SF_6 was not blown close to the subject, and as soon as possible, the wash-in gas was turned off.

Wash-out Phase

The patient was then left, breathing room air, until the maximum concentration of SF_6 displayed on the screen was 0.003% for 3 successive breaths. The mouthpiece was then removed, and the patient offered a glass of water and a short break.

The test was repeated three times, or more if any of the tests were thought likely to be technically inadequate, e.g. due to coughing, unsteady breathing or inappropriate disconnection at any point.

Problems encountered

Very few technical problems were encountered when performing MBW tests. The most common problems included: failure of the Innocor gas analyser, disruption to the breath trace caused by the subject coughing/ moving and imprecise disconnection of the tracer gas (Figure 2.2-2).

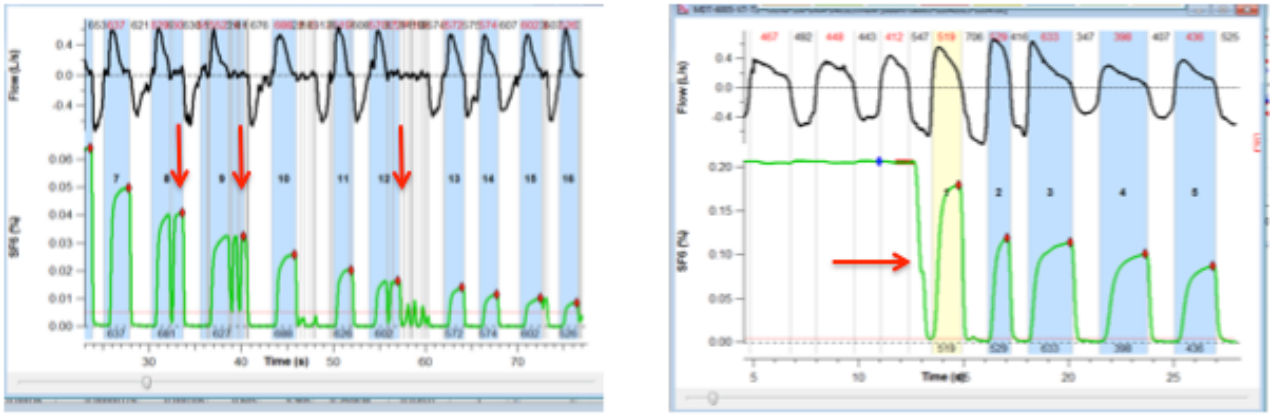


Figure 2.2-2: Illustration of problems encountered when performing MBW traces using SF_6 and the Innocor device with SF_6 on y axis and time on x axis a) trace disrupted by a coughs (indicated by the 3 red arrows) b) imprecise disconnection of the tracer gas (indicated at the horizontal red arrow)

Data export

Raw data from the Innocor was exported to a .ino file on a USB memory stick before switching off the Innocor to ensure no data was lost. The file could then be uploaded for analysis.

2.2.3.5 Calculation of LCI

To obtain the final values for LCI, the SimpleWashout software (NB, University of Edinburgh) was used. This programme was written using the IGOR Pro (WaveMetrics Inc) data analysis platform. Data files were imported into the SimpleWashout software by converting the .ino file to .txt file containing flow, SF_6 and CO_2 data. The following data inputs were required:

- Setting the disconnection point and grading the quality of the disconnection (ensuring it had not taken place during and the inspiratory phase)
- The cursors for C_{max} (concentration of SF_6 at the end of the last inspiration before disconnection), and C_{init} (average concentration of SF_6 at the end of the last expiration before disconnection) were automatically placed on the trace by the software. If the concentration of SF_6 varied even

slightly just before the disconnection these may be placed in the wrong part of the trace and require manual correction by eye.

- Verifying that the first breath and subsequent breaths had been correctly identified
- Checking the washout termination status
- Checking the SF_6 offsets

The SimpleWashout software allowed an offset correction for any SF_6 gas present in the room at the beginning of subsequent tests. The results from 'Offset corrected' LCI measurements were used to represent the final value for LCI.

2.2.4 Nasal brush biopsies (Chapters 3,6)

The nasal brushings obtained were performed as according to the Royal Brompton Hospital NHS Trust Electron Microscopy Unit - Primary Ciliary Dyskinesia diagnostic service SOP. The subject cleared their nose of mucus by gentle blowing, and sat down on a chair positioned against the wall, with their head reclined. A disposable cytology brush (Olympus) was inserted into the nose, beneath the inferior turbinate, using an otoscope for guidance. The ciliated epithelium in a single nostril was brushed against the inferior surface in an anterior-posterior motion with a degree of rotation for 5 seconds to collect strips of ciliated epithelium. Cells obtained were suspended in bronchial epithelium growth medium (BEGM) containing the antibiotics penicillin (100IU/ml) and streptomycin (100µg/ml) (PS) and stored immediately at 4^{o}C.

2.2.5 Assessment of inflammatory cytokine concentrations (Chapters 5,6)

Samples of cell suspension, sputum, supernatant from cell culture and nasal epithelial lining fluid (NLF) were collected for analysis of the cytokine profile. Details on sample collection are described in the relevant chapters. 2 different platforms were used in this thesis to measure inflammatory cytokine concentrations. The IL-8 ELISA (ThermoFisher SCIENTIFIC (KHC0081)), and the Human ProInflammatory 9-plate Tissue Culture Kit (Meso Scale Discovery (MSD) platform) (K15007B-1), designed to quantify the presence of GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, and TNF-α in tissue culture supernatant. This plate was chosen as these cytokines play a role in pivotal role in the inflammatory response and have been shown to be elevated in the airway during infection (1.3.2.3). Both methods are described here.

2.2.5.1 IL-8 ELISA

Each analysis was performed as per manufacturers guidelines [255]. 50μL samples of the sample and the control substance were added to microtitre wells prepared as per the SOP. 50 μL of the anti-IL-8 solution was added and the wells gently mixed and incubated. The solutions were then aspirated and the wells washed. 100μL of Streptavidin-HRP working solution was then added, and the wells incubated again. Finally, the wells were washed again, and 100μL of *Stabilized Chromogen* was added. The solution was incubated for a pre-determined time and then 100 microlitres of *Stop Solution* was added. The optical density of the wells were then read at 450nm using a plate reader that had been blanked against a well containing only *Stabilized Chromogen.*

2.2.5.2 MSD platform

The MSD plates contain 96 wells, on the bottom of which is a multi-array plate. The plate is pre-coated with the selected specific cytokine capture antibodies of interest. The sample is added to the well in addition to a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) and incubated for a defined period. Analytes in the sample bind to the capture antibody, which is immobilized on the electrode surface, and to the detection antibodies (Figure 2.2-3). MSD read buffer is added to the wells, which provides the appropriate chemical environment for electrochemiluminescence. When the plate is loaded into the MSD imager, a voltage is applied to the plate electrodes causing the MSD SULFO-TAG™ to emit light. The imager measures the intensity of the light emitted to provide a quantitative measure of analytes in the sample.

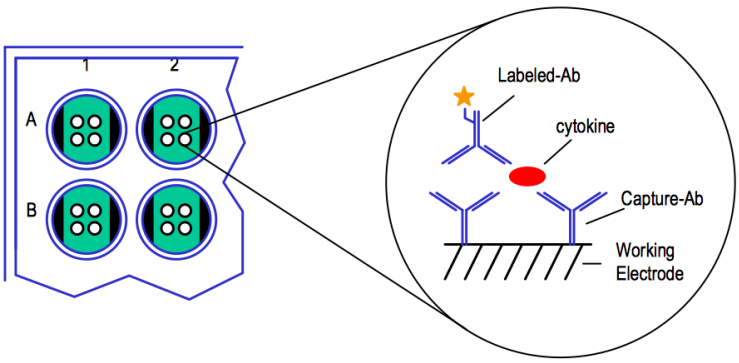


Figure 2.2-3: Immunoassay format using capture antibodies on the MSD multi-array plate, illustrated on a 4 spot MSD MULTI-SPOT plate. Reproduced from website [256]

Method

Preparation

- A calibration curve was created by diluting the stock calibration solution sequentially in a diluent containing a standard tissue culture growth medium with 10% serum. The calibration samples placed into the first 2 columns of the MSD plate (step 1). This created a standard curve ranging from 2.4ρg/ml to 10000ρg/ml.
- The Detection Antibody Solution was prepared by diluting 60uL of the Detection Antibody Mix into 2.94ml of the provided diluting agent. This was kept in the dark, as some antibodies are light sensitive.
- 10 ml of the Read Buffer were diluted in 10ml de-ionized water.

Steps

1. 25μL of each test sample or the calibration sample was dispensed into a separate well of the plate. The plate was sealed with an adhesive plate seal for 1-2 hours, shaking at 300-1000rpm) at room temperature.
2. 25μL of Detection Antibody Solution was dispensed into each well of the MSD plate and again sealed an incubated for 1-2 hours at room temperature, shaking 300-1000rpm)
3. The plates were washed 3 times with PBS +0.05% Tween-20 followed by the addition of 150μL of the Read Buffer solution to each well of the plate.
4. Plates were read immediately using a a Bio-Rad Bio Plex 200 plate, after the addition of Read Buffer

The results were generated on the plate reader and transferred to excel files for further analysis. The calibration curves were verified for each cytokine. When available, duplicate values were compared and the coefficient of variation calculated. None of the values obtained were above the upper limit of the calibration curve. Many values were recorded near or below the limits of detection. The results have been analysed however and this is discussed in the relevant sections.

2.2.6 Sweat test and analysis (Chapters 3,6)

Sweat tests were performed using the Macroduct (R) Sweat Collection System and were performed in accordance to the manufacturer's instructions [257].

A selected area on the patient's forearm was cleaned and 2 electrodes with Pilogel (pilocarpine gels) discs installed were attached to the forearm with velcro straps, coloured to indicate positive and negative. The electrodes were attached to the Webster Sweat Inducer, which activates iontophoresis, for 5 minutes. The electrodes were then removed and a Macroduct Sweat collector firmly attached to the stimulated skin site, where the positive electrode had been attached. The sweat was collected along the spiral tubing; a blue indicator dye allows this to be visualised. When the collection was complete, tubing was severed from the Macroduct base and the sweat specimen was collected into a universal container tube and sent to the Biochemistry lab at RBH, for analysis of the sweat chloride concentration. This process was performed on both arms, and the mean of both measurements taken as the value for the patients' sweat chloride concentration [258].

Very few technical problems were encountered. On several occasions insufficient sweat was collected, requiring a repeat of the test.

2.2.7 CFTR mutation analysis (Chapters 3,4,6)

CFTR gene mutation analysis was not repeated if it had previously been performed for clinical reasons and the information was available.

Blood samples were taken from patients and were sent to the Kennedy Galton Genetics Lab and analysed for the presence of any genetic mutations known to cause CFTR dysfunction. Analysis was performed using the Devyser CFTR- Core Kit, which includes a panel of the 36 most common mutations found in the European population [259].

2.2.8 Airway potential difference measurements (Chapters 3,4,6)

This section describes the methodology for measurement of airway potential difference.

2.2.8.1 Nasal measurements

Equipment:

The solutions; Ringers solution, Ringers Amiloride solution and Zero Chloride Amiloride (ZCl/ Amiloride) solution, were prepared at the Eastbourne Hospital Pharmacy and supplied in 50 ml glass bottles in batches (composition in Appendix A). At the RBH they were stored at 4^{o}C.

- Stock Ringers solution
- Stock Ringers Amiloride solution
- 2 x Stock Zero Chloride Amiloride
- 11 x19G needles
- 4x 50 ml, 2 x 5ml ,1 x 2ml, 1 x 1ml syringe
- 2.25mg/2ml vial pack Isoprenaline
- Marquat Dual-Channel Nasal Probe – Length 80cm; Diameter 2.5mm (Ref: I0202US)
- Alaris pump x 4
- 1 x 2ml syringe and 2x 5ml syringes
- Alaris microtubing (MFX2259)
- 3- way tap x 3 (3 for tubing circuit, 1 on catheter)
- T-piece connector x 4
- Laptop computer with PD software (Logan Research Ltd)
- LR4 millivoltmeter (Logan Research Ltd)
- Silver-silver chloride electrodes (Biosense Medical) x 2
- Electrode cream (Signacreme, Parker laboratories)
- Dental drill system including sterile single use dental burr
- Sleek (tape)
- Steristrips
- Micropore tape 70%
- Alcohol Skin Swab (Steret or equivalent)
- Sterile single pack yellow pipette tip
- Small forceps

- Chin rest
- Cardboard disposable tray / bowl
- Paper towels

Equipment Preparation

Preparing the solutions and tubing circuit is illustrated in Figure 2.2-4.

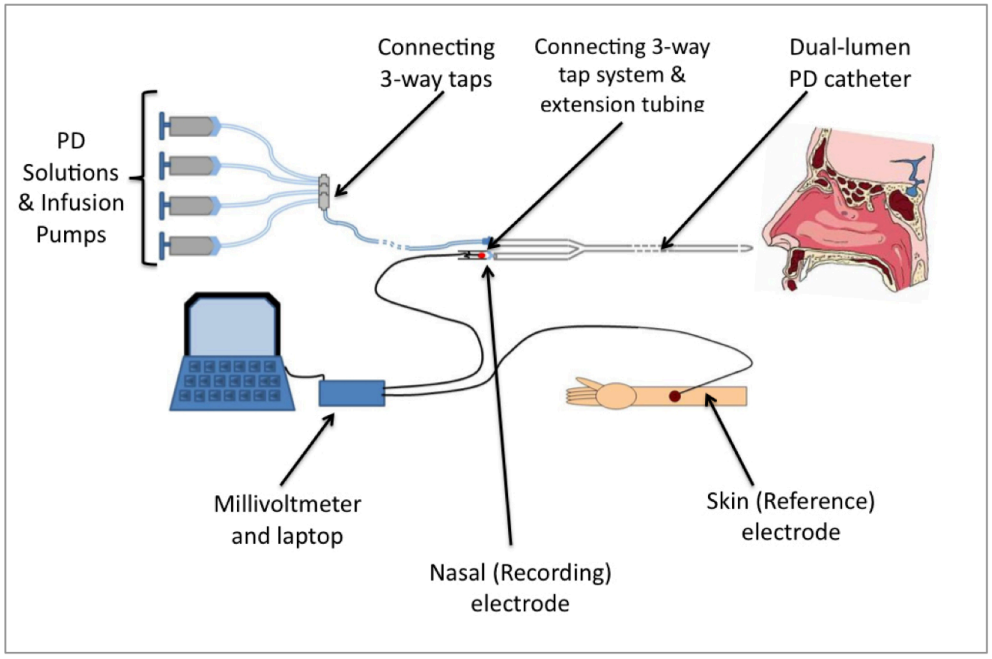


Figure 2.2-4 Schematic diagram showing the set-up of NPD equipment, as described in the following paragraph (image courtesy of Dr Michael Waller)

The solutions were allowed to warm to room temperature, then drawn up into 50ml syringes and the syringes labeled to identify the contents. To prepare the ZCl and Isoprenaline (ZCl/Iso) solution, 0.11ml of Isoprenaline (2.25mg/2ml) measured in a 1ml syringe, was added to a 50ml syringe of ZCl/ Amiloride solution and shaken to mix to a final concentration of 2.5 mcg/ml. This solution was used within 2 hours of adding the isoprenaline to ensure it did not deactivate. The four syringes were placed in syringe drivers in the order in which they were to be perfused. A t-piece was attached to the end of each of the solution syringes, and the t-pieces connected to each other with three 3-way taps.

Setting up the computer equipment

The Voltmeter was attached to a laptop computer via the USB connection. 2 electrodes were attached to the Voltmeter with the red port connected to the recording (nasal) electrode and the black port connected to the reference (skin) electrode. The Voltmeter, followed by the computer, were switched on and the Logan Software programme opened. Initially the scale of the graph was changed to -80 on the x axis, and the programme set to 'run'.

Off set

Both electrodes were placed in a sterile container filled with electrode cream, and the trace monitored until the values were stable and <+/- 5mV. If the recording was outside this range, a new set of electrodes were chosen and measured. When satisfactory, the offset was recorded, and the 'offset correction' option chosen from the menu to reset the trace to zero.

Preparing the nasal catheter

For each patient a new, Marquet double lumen catheter was used. 2ml of electrode cream (SIGNACREME® electrode cream) was injected into the catheter through the luer-lock port through the channel marked with a yellow band until a steady flow of cream was seen exiting the catheter. A 5 ml syringe, half filled with electrode cream, with the plunger removed, was connected to the yellow band marked port. The recording (nasal) electrode was then inserted into this 5ml syringe. The offset was rechecked to test the electrical circuit of the nasal catheter in the Electrode Cream and corrected if >0.5mV. The reference electrode was then secured in the syringe using adhesive tape.

Performing measurements

A proforma was used to record information during the procedure. This included both pre- and post-procedure electrode offsets, skin PD measurement, subject's clinical status, any unexpected events or problems, and the timings that the solutions changed and the corresponding PD values.

Measuring Skin PD

The subject's skin was cleaned with an alcohol wipe, and a shallow skin abrasion made using a sterile, single-use dental drill burr. A piece of sleek was placed over the reference electrode (skin),

and a small hole made in the tape overlying the circular hole in the electrode. This was then placed exactly over the abraded skin site. A small syringe was filled with electrode cream, attached to a 21G needle, inside a yellow pipette tip. Electrode cream was then injected through the hole in the sleek, providing contact between skin and the electrode.

The catheter, ready primed with conducting cream, was placed on the patients' fingertip and the value for the skin, displayed on-screen, was documented. This confirmed the integrity of the electrical circuit and that skin contact was adequate. Skin measurements generally lie between -30 and -60 mV, and if skin PD was <30mV the circuit was reviewed and the forearm skin preparation process repeated. Patients with CF have a typically more negative skin PD.

Measuring nasal PD

The patient rested on a chin rest to help maintain head stability during the procedure. With the head in neutral position, the catheter was inserted slowly along the nasal floor, with the blue marker on the catheter pointing to the left side of the operator to ensure that the recording port was in contact with the floor of the nostril. The site with the highest (most negative) PD, after testing both nostrils, was chosen to be the measuring site, and the length of the catheter into the nasal cavity and which nostril used was documented. For subsequent measurements in the same subject, the same site was used. The electrode was held in place using steristrips, and the head was positioned downwards prior to starting the perfusion of the solutions.

The syringe pumps and the computer were disconnected from the main electricity supply, and powered by battery to reduce electrical interference. Perfusion of the first solution was commenced at a flow rate of 4ml/minute. The solutions perfused were:

- Ringers solution until the trace was stable (minimum 30 seconds)
- Ringers + Amiloride (minimum of 3 minutes and the trace was stable)
- ZCl + Amiloride solution (5 minutes)
- ZCl + Amiloride with Isoprenaline solution (5 minutes)

When the solutions were changed, the PD value displayed on the screen was documented and the Voltmeter switch moved from O/P to CAL to O/P to place a mark on the screen. The solution was stopped and the 3-way tap switched to allow the next solution to run. A typical trace of a healthy control and CF patient were illustrated in Chapter 1 Figure 1.3-1.

Problems encountered:

Equipment failure: Equipment failures predominantly involved the presence of bubbles in the electrode cream in the catheter circuit which interrupted the electrical circuit and caused disruption to the trace (frequent). Other problems included displacement of the nasal catheter during the procedure either due to poor securement or the patient moving secondary to coughing or talking (occasional).

2.2.8.2 Adaptation of PD measurements for lower airway

All measurements were performed with patients under a general anaesthetic (GA) administered by Dr Brian Keogh, Consultant Anaesthetist RBH. The bronchoscopy was performed on all occasions by Professor Jane Davies (Professor of Paediatric Respirology and Experimental Medicine, Imperial College) and LAPD measurements by either myself, or my colleague Dr Michael Waller.

Equipment list

- Flexible Bronchoscope
- Biopsy forceps (Disposable biopsy forceps, Olympus, FB-231D) x 1 per bronchoscopy
- Stock Ringers and Zero Chloride Solutions, in 50 ml pre-prepared bottles.0.22micron filter, vial of Isoprenaline sulphate 2.25mg/2ml, 1ml syringe, green needle, 50ml syringes, syringe to syringe transfer bung, bungs and worksheet as prepared by pharmacy.
- 5ml syringe containing (solid) 1ml 4% Agar in 3M Potassium Chloride.
- Alaris syringe pump x 2
- Silver-Silver chloride Skin electrode (Biosense Medical) labelled with study ID number
- Silver-Silver chloride measurement electrode (Biosense Medical)
- 18G Abbocath-T x 2
- T-piece connector x 3
- White (19G) needle x 2
- 2ml syringe and 5ml syringe
- Signacreme
- Dental drill system including sterile single use dental burrs
- Sleek (tape)
- 70% Alcohol Skin Wipe (Steret or equivalent)
- Sterile single packet Yellow pipette tip
- Green needle (21G)

- Laptop with PD software (Logan Research Limited)
- LR4 millivoltmeter (Logan Research Ltd)

Solutions:

Ringers and Zero Chloride (ZCl) (no amiloride) solutions are prepared and stored as per Appendix A.

Equipment Preparation

Figure 2.2-5 shows a schematic representation of the LAPD set up as it was used in the gene therapy Multidose Trial.

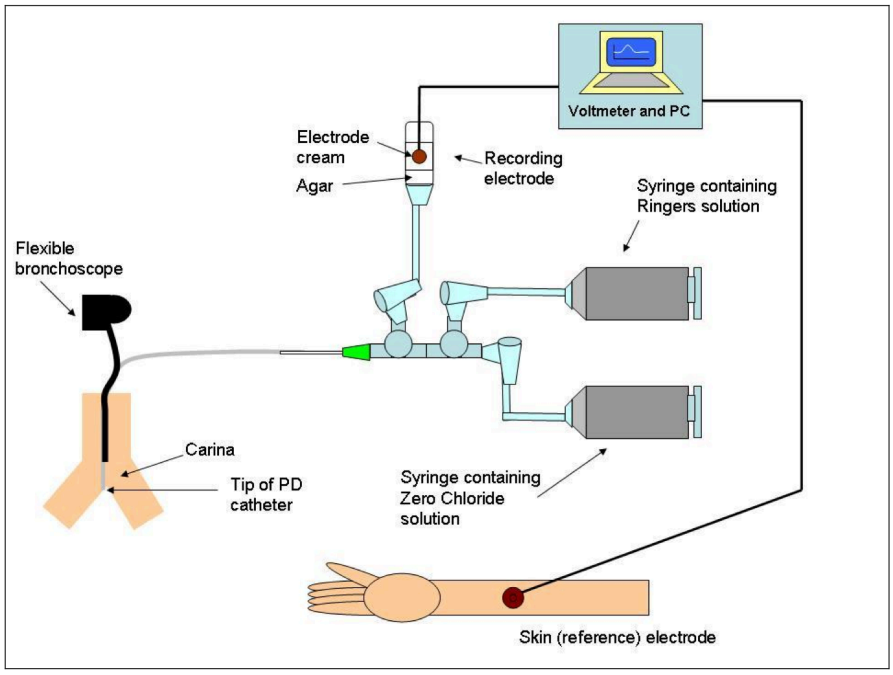


Figure 2.2-5: Schematic diagram of the LAPD set up, (image courtesy of Dr Gwyneth Davies)

The day prior to the procedure:

- 2 x 50ml Ringers Solution bottle and 1x 50ml ZCl were placed in the fluid warmer at 37^{o}C; this meant that the solutions were cooling down during the set-up reducing micro-bubble formation.

- The agar syringe was made. 0.4mg of agar powder was added to 10 ml of potassium chloride solution and gently heated to ensure it dissolved. 5 ml syringes, with the plunger remover, were filled carefully with 2 ml of agar, ensuring that it was present in the hub of the syringe and absent of air bubbles. These were left to set overnight.

The day of the bronchoscopy:

- All solutions were removed from the warmer and 50 ml of both Ringers solution and the ZCl solution were drawn up into a 50ml syringe.
- ZCl/Iso was made up; as described for NPD measurements.

Circuit

A t-piece was attached to the end of the Ringers solution syringe and primed with the solution. Any bubbles trapped in the t-piece were displaced by gently tapping the tubing. A second t-piece (2), also primed was attached to the first t-piece, and t-piece 1 clamped off. The rubber bung of the t-piece (1) was pierced with a 19G needle whilst gently purging the syringe allowing the needle to fill with Ringers solution. When a meniscus of fluid was visualised on the top of the needle hub it was attached to the agar filled syringe. Approximately 2ml of SIGNACREME® electrode cream (Parker, USA) was added on top of the Agar in the syringe. A third t-piece (3) was attached to the ZCl solution syringe, and primed carefully to ensure no bubbles were present. The 'off' bung of the 3-way tap was placed on the t-piece (2) and the rubber bung pierced with a 19G needle and filled with Ringers solution as before. The primed t-piece (3) was then connected to this needle and clamped off. The syringes were then placed carefully in the pumps and the circuit was secured to the side of the pump with sleek tape.

For the PD catheter, the sheath surrounding a disposable cytology brush was used, into which an 18G Abbocath catheter was inserted roughly 4mm. The green end of the Abbocath was attached to the remaining port of the t-piece (2) followed by purging the Ringers solution from the infusion pump to prime the Abbocath and the PD catheter.

Setting up the computer

The only difference between the set up for NPD (section x) compared to LAPD is the selection scale of the y axis on the graph was selected to -40mvol.

Offset

A second 18G Abbocath was inserted into the end of the PD catheter (to preserve sterility of the latter) and the needle removed and disposed of. Ringers solution was purged to the end of the Abbocath until a meniscus was seen at the green open end. The measurement electrode (Red port) was placed in the syringe containing electrode cream above the agar, and both the reference (skin) electrode and the green end of the Abbocath were placed in a Universal container containing Signacreme. The offset was measured and corrected for as described above.

Performing measurements

A proforma was used to record information during the procedure; including pre- and post-procedure electrode offsets, the skin PD value, comments about the general appearance of the airways and the side chosen from which to perform the measurements. The same side was used both pre- and post- dosing. The timings that the solutions changed and the corresponding PD values were documented, in addition to any unexpected events.

Measuring Skin PD

Performed as above for NPD measurements

Measurements in the airway

LAPD measurements were performed as listed below.

- The bronchoscope was introduced
- The lung with least inflammation and mucus was chosen to perform the PD measurements to maximise contact between the electrode and bronchial epithelium.
- The PD catheter was inserted into the bronchoscope and the tip of the catheter rested against the lower airway mucosa.
- Ringers solution was connected and perfused onto the mucosa at a flow rate of 100ml/hour. Measurements were taken at 4 locations at the level of the carina: anterior, posterior, right lateral and left lateral.
- Ringers solution was run through the catheter at each location until a stable measurement was recorded, (minimum 20 seconds).
- The bronchoscope was repositioned to a distal site (approximately 5th generation bronchi).

- Ringers solution was perfused onto the mucosa until values were stable (minimum 20 seconds).
- The solution was changed to ZCl/Iso.
- The stop clock was started for 300 seconds
- At 300 seconds, ZCl/Iso was stopped, the sheath withdrawn, and purged with 2ml of Ringers solution to eliminate the ZCl/Iso solution from the catheter.
- The procedure was repeated at 2 further locations in the distal bronchus in the same lung.
- The offset of the electrodes was rechecked, and documented, to ensure there had been no significant drift in the voltage measured.
- Bronchial brushings and biopsies were performed at the end of the procedure as described in Chapter 4.
- A single dose of two appropriate antibiotics was administered following the procedure to limit any potential adverse effects.
- When woken from the GA, patients were monitored for a minimum of 4 hours before discharge.

A typical LAPD trace from a subject with CF is illustrated in Chapter 4 Figure 4.4-2, along with examples of traces that were difficult to analyse due to problems encountered.

Problems encountered

Loss of electrical contact due to visible bubbles, or micro-bubbles in the circuit: on several occasions the electrical trace was lost as evidenced by a sudden change in the PD measurement. On these occasions the catheter was purged with the solution that was currently running to dislodge the bubbles until contact was regained. If no contact was regained the trace was discontinued.

Agar syringes dislodged: On 2 occasions the agar disconnected from the syringe. The syringe was immediately replaced with a spare and the procedure was continued. Agar syringes were made at least 18 hours before the procedure to allow time for them to solidify completely, and several were made at one time to provide a replacement if required.

Medical concerns: If the subject encountered any medical problems (desaturations, hypercarbia or haemoptysis) during the procedure, it would be discontinued and only restarted if considered to be safe by all medical members present. One patient experienced an episode of hemorrhage following the endobronchial brushing, which resolved spontaneously but the procedure was discontinued.

Saving airway PD data

Files were saved to the computed in a Logan prop.binary data file which was 'DataDumped' to create a compatible text file which was used for the data analysis.

2.2.8.3 Analysis of airway PD measurements

Nasal PD measurements

The nasal PDs were analysed as according to the UKCFGTC analysis of NPD measurement SOP. The text files containing the PD values were transferred to an Excel worksheet for further analysis. The values were then converted into a graph so that the trace could be visualised.

Measuring the phases of NPD in Excel:

The transition point between the NPD phases was mostly clearly visible due to the marker places on the trace by the operator at the time of the procedure. If this was not obvious, the time written in the proforma was used.

Baseline/ Ringers: The time at which the Ringers phase was completed was visualised. The values for the 20 seconds preceding the transition were averaged to provide a basal value.

Amiloride response: To determine the voltage after perfusion of amiloride, the same process was repeated as for the Ringer's phase. The amiloride response is the Ringers value - the Ringers amiloride value

ZCl/ Amiloride: This value was measured at 300 seconds from the changeover point.

ZCl/ Amiloride + Isoprenaline: same process was repeated as for the ZCl/ Amiloride phase.

Total chloride response: ZCl/ Amiloride response + ZCl/ Amiloride + Isoprenaline response

All delta values (the amiloride response and the both the ZCl phases), values were calculated from

the start of each specific NPD phase but before 30 sec, therefore excluding any electrical artefact that occurred in the PD trace during the changeover of solutions and the influence of the subsequent perfusate on the epithelium.

Lower airway PD analysis

This is described in Chapter 4

2.2.9 Statistics

Data were analysed using Prism 6 statistical software (GraphPad Software Inc, CA, USA). The specific statistical tests used in each chapter are described in the relevant sections. Normality of the data was determined using the Kolmogorov-Smirnov test. Where data were non-parametric, paired comparison pre treatment and post treatment values were analysed using the Wilcoxon matched-pairs Signed Rank Test. Comparison between active and placebo groups was assessed with Mann-Whitney U test (normality not assumed due to small data size). Correlation between outcome measures was evaluated in these data sets using non-parametric spearman rank correlation. The null hypothesis was rejected at $p<0.05$.

Where multiple sets of data were compared to each other, the non-parametric Kruskal-Wallis test was used. The Dunn multiple comparison test was performed to determine whether the post-hoc tests were significant.

3 Gene Therapy Multi Dose trial

3.1 Introduction

Alongside the development of small molecular therapies for CF, there have been significant advances in gene therapy for CF, which aims to correct the underlying genetic abnormality itself [260]. The UK CF gene therapy consortium (GTC) conducted a gene therapy Multidose Trial, designed to assess for the first time whether the administration of repeat doses of lipid-mediated gene therapy to the lungs of patients with CF results in a clinical benefit, rather than, as in other gene 'therapy' trials, whether WT-CFTR can be expressed in the airway. The primary outcome was relative change in FEV1, but the opportunity was also taken to explore CFTR surrogates including: LAPD measurements performed on a subgroup of patients in this trial both pre- and post- gene therapy dosing. The Multiple dose trial provided the unique opportunity for this thesis both to evaluate LAPD measurements as a marker of CFTR function in subjects with CF and to further explore the relationship between CFTR protein function and airway disease.

This introduction provides a summary of the recent advances in gene therapy in CF leading up to the Multidose Trial. This chapter describes the trial in detail and provides context for Chapter 4, which describes the LAPD-focused study and the correlation between these measurements and the markers of airway inflammation, in order to address the underlying hypothesis; that here is a direct relationship between CFTR function as manifest by sodium and chloride transport and airway health.

3.1.1 Gene therapy for CF

CF is considered to be an appropriate target for gene therapy as it is a single gene disorder, and the airways, which carry the majority of disease burden, are relatively accessible for topical non-invasive drug delivery. Furthermore the CF airways are thought to be unaffected at birth suggesting that there is a potential therapeutic window. Gene therapy can be achieved by either gene replacement therapy, or by gene editing techniques. The latter involves correction of genetic defects in-situ, via DNA or RNA editing, and is an emerging therapeutic avenue in CF [261]. This introduction focuses on gene replacement, in which the aim is to transfer a functioning copy of CFTR into the airway epithelium, which is transported into the cell nucleus, allowing transcription of the therapeutic gene. The airway however, has developed physical and immunological barriers as defence mechanisms (described in section 1.1), in addition to cellular barriers to cell entry and

access to the nucleus, all of which must be overcome in order for gene therapy to be successful. Vectors are required to assist the entry of DNA in to the airway epithelial cell, of which the most extensively used in trials to date are either genetically modified viruses, or non-viral vectors such as liposomes.

3.1.1.1 Clinical trials in gene therapy in CF

Viral vectors

Viruses were considered suitable vectors as they have naturally evolved to evade many host defences and achieve effective cell entry. Early studies focused on the use of adenovirus (AV) and adeno-associated viruses (AAV). The first clinical trial in gene therapy in CF in 1993 used an AV-vector coupled with WT-CFTR, which was instilled on the nasal surface of 3 CF patients [262]. CFTR mRNA and protein were undetectable, however NPD measurements demonstrated a return towards normal baseline and chloride response, providing 'proof of concept', which then led to a number of larger trials. The majority of these delivered gene therapy to the nasal mucosa as a surrogate for the lower airways for safety reasons in addition to the technical advantages in drug delivery and tissue sampling. Changes in NPD measurements, presence of CFTR mRNA and CFTR protein, have been used most commonly as evidence of successful gene transfer [263-265]. Together these trials demonstrated that the products were safe and well tolerated, and provided variable evidence of successful gene transfer, typically in the order of 20-25% correction of the chloride defect.

The first study looking at the response to repeat AV-mediated gene therapy administered 5 doses to 6 CF patients [266], demonstrating a partial chloride defect correction in some subjects. However, there was less correction of the defect with each subsequent administration, thought to be due to the development of a host immune response to the vector preventing gene transfer on subsequent doses, although no patients developed antibodies to CFTR itself. This has consequently been shown to be a major limitation to the use of the early viral vectors [267-270]. As CF is a life-long condition, unless gene therapy is able to target stem cells, it must be repeatedly delivered in order to produce a long-term clinical benefit. The feasibility of stem cell based therapy has been explored, however to date both in vitro and animal models have not been promising. As the target cells (bone marrow) are not easily accessible, delivery of CFTR must be systemic, and studies have demonstrated that this has limited success in trans-differentiation into the airway epithelium [271]. In gene therapy, there has only been 1 trial using a viral vector, designed to assess clinical improvement. The initial safety arm of the study demonstrated that some subjects

showed a small but significant improvement following 3 doses of nebulised AAV mediated CFTR cDNA 30 days apart, however these results were not replicated in the larger phase IIb study [268, 272].

Non viral vectors

Trials of non-viral vectors commenced as a response to the immune response to viral vectors limiting the effectiveness of repeated gene transfer. The most extensively trialed agents currently are cationic liposomes, which achieve cell entry by endocytosis across the cell membrane. Initial non-viral vector studies looked at nasal administration in small numbers of CF patients. These showed evidence of *CFTR* expression, including partial correction of the chloride defect detected by NPD lasting from 3 days up until a month in some patients. Administration was well tolerated without adverse effects and significantly, the delivery of 3 doses at 4 weekly intervals did not produce an immune response or result in reduced efficacy with subsequent doses, thus supporting its role in further trials for long-term treatment of CF [175].

The first clinical trial administering lipid-DNA complex to pulmonary mucosa compared the responses of 8 patients receiving nebulized doses of the cationic lipid (GL67) coupled to *CFTR* under the control of a cytomegalovirus (CMV) promoter, followed one week later by a nasal dose, with 8 patients receiving lipid alone [194]. Results revealed approximately 25% restoration of chloride channel function which persisted for 3 weeks, accompanied by a significant reduction in sputum inflammatory cells, and reduced bacterial adherence on epithelial cells obtained by bronchial brushing. Side effects of the active treatment included a transient drop in respiratory function, and mild flu like symptoms that had resolved by 48 hours. Similar to previous trials, no reduction in sodium transport in NPD measurements was seen [194]. *In vitro* studies have shown that change in chloride secretion can be achieved following CFTR correction in a minority of cells whereas correction of Na^+ hyperabsorption requires normal CFTR function in almost all cells [273]. It is currently unknown whether correction of sodium transport, in addition to chloride is required for clinical benefit [274].

3.1.2 UK Gene Therapy Consortium

The GTC was founded in 2001 with the aim of sharing expertise and to coordinate the development of gene therapy for CF in the UK, linking scientists and clinical sites in Edinburgh, Oxford and London. There have been two focuses: working towards the gene therapy Multidose

Trial (Wave 1), and the development of a modified lentiviral vector for clinical trials (Wave 2); discussed under future work (Section 3.5).

3.1.2.1 Gene therapy Multidose Trial (Wave 1)

The GTC has been working towards performing a large clinical trial with the aim of delivering multiple doses of lipid-mediated gene therapy over a long enough period to produce clinical benefit, delivered via a nebulizer to the airways of patients with CF.

The gene therapy product was optimised prior to the trial to ensure that it was the most effective, safe and well tolerated available. The side effect (a flu-like inflammatory response) was thought to be due to an innate immune response to the un-methylated CG dinucleotide (CpG) motifs present in the plasmid DNA. A CpG-free plasmid was developed which was shown in preclinical models to eliminate the inflammatory response [275]. In addition, the CMV promoter utilised in previous trials, which had a rapid onset of action but silenced quickly, was changed to a humanised promoter coupled with the elongation factor 1a promoter (hCEFI), to increase the quantity and duration of gene expression. The final product is called pGM169, which was shown to provide efficient gene transfer into both human airway cells and animal models, without provoking an immune response on repeat administration [276, 277].

Pilot study:

A single-dose safety pilot trial was performed with several aims; to confirm safety following a single dose of gene therapy, to assess for evidence of successful gene expression, to determine the dose for progression into the Multidose Trial, and assess the duration of gene expression. Patients received a single dose of nebulised pGM169/GL67A GT and/or a dose instilled onto the nasal mucosa (nasal dose). Three volumes of airway doses were evaluated; 5ml, 10ml or 20ml and measurements of safety and gene transfer and expression were recorded at 1,2,6,14 and 28 days following the dose. Safety assessment included clinical observations and physical examination, adverse event recording, spirometry, HRCT, and serum inflammatory markers. Gene expression was evaluated using PD measurements in the nasal and lower airway mucosa and detection of transgene mRNA from cell brushing. The 5 ml dose was shown to be optimal; with less systemic inflammatory response and reduction in pulmonary function than seen at larger doses whilst demonstrating changes in CFTR molecular surrogates [278].

Run-in Study:

The GTC performed a 'Run-in' study; a 3 year multicentre trial, conducted to identify which outcome markers best assessed any clinically relevant response to gene therapy, and which patients to take part in the Multidose Trial [279]. 192 patients with stable CF (aged>10 and FEV_1>40% predicted) were enrolled. Spirometry and LCI were performed, sputum and blood samples were taken for assays assessing inflammation, and radiographic images were obtained at 4 time points over the first 12-18 months, then at 6 monthly intervals. FEV_1 % predicted was chosen as the primary outcome, as it was shown to be the assay requiring the fewest patients for adequate statistical power, and is the most acceptable outcome measure in CF to regulatory agencies. Amongst other measures, CT chest, quality of life questionnaire and LCI were included as secondary outcomes. Suitable patients were identified as >12 years old, with FEV_1 50-90%. These parameters were chosen as patients with lower lung function have inflamed airways with large volumes of mucus, obstructing contact between gene therapy and the epithelium [280]. In patients in whom FEV_1 was already >90%, it is difficult to demonstrate an improvement.

3.2 Gene Therapy Multidose Trial – Method

3.2.1 Aim of the trial

To assess for the first time whether administration of repeat doses of lipid-mediated gene therapy to the airways of patients with CF results in a clinical benefit.

3.2.2 My role in the trial

The Multidose Trial had been running for 4 months prior to my commencing this MD(Res). As one of 2 clinical research fellows at the RBH site I took on a pivotal role in the everyday running of the trial, including;

- Lead for recruitment of the paediatric patients at the RBH
- Assisted in setting up the UK wide Participant Identification Centres (PICs).
- Consented both adult and paediatric patients,
- Provided medical care and assessment.

- Either myself, or my colleague, performed all NPD measurements, nasal brushings, and blood sampling,
- I was part of the team who performed the LAPD measurements.
- I helped amend the trial protocol and patient information sheets (PISs), wrote SOPs
- As a team of two, we assessed and graded all of adverse events (AEs) from the London site.
- Following the completion of the clinical trial visits I assisted in the analysis of the MBW traces for LCI, trial data entry and monitoring the quality of data input.
- I jointly analysed all LAPD traces and assisted in developing methods to analyse and interpret the results as part of a group consisting of Professor Eric Alton, Professor Jane Davies, and Dr Michael Waller.

3.2.3 Study personnel

The Chief Investigator was Professor Eric Alton. Trial visits took place in London, at the RBH, and in Edinburgh, at the Royal Hospital for Sick Children or the Western General Hospital. The clinical team in London, consisting of 2 trial doctors, 3 nurses, 2 physiologists and a clinical trial coordinator and was led by Professor Jane Davies. The clinical team in Edinburgh; consisting of 2 trial doctors and a clinical trial coordinator was led by Dr Alistair Innes. The scientists and laboratory technicians at London and Oxford were led by Professor Uta Griesenbach and Dr Steve Hyde, respectively.

3.2.4 Subjects and recruitment

The trial ran from June 2012 to May 2014 and recruited patients aged >12 years with CF, defined in Section 2.1. Patients who had taken part in the 'run in' study, and who were eligible were invited to take part. Following this, potentially eligible patients receiving their medical care from any of the 3 trial sites, were identified from clinic lists and electronic records. In December 2013, PIC sites were included and patients from any of the collaborating sites across the UK could also be referred by their local team to participate. Subjects were approached by telephone or during a clinic visit and followed up by telephone. Interested subjects attended an initial visit to find out more about the trial. Consent (and assent if the patient was a child) was performed by one of the clinical trial doctors and the subject was enrolled if they fulfilled the inclusion criteria as shown in Table 3.2-1

Inclusion criteria
CF confirmed by sweat testing or genetic analysis
Males and females > 12 years old
FEV_1 between 50 and 90% inclusive (Stanojevic reference equations)
Clinical stability at screening
All patients were required to take effective contraception for duration of the trial and 3 months after
Withhold DNAse for 24 hours before and 24 hours after the GT dose
Written informed consent obtained
Permission to inform GP of participation in study
Exclusion criteria
Infection with *Burkholderia cepacia* complex organisms, *MRSA* or *M abscessus*
Significant nasal pathology including: polyps, clinically significant rhinosinusitis, severe epistaxis (nasal cohort only) Chloride secretory response on nasal PD of >5mV (nasal cohort only)
Acute upper respiratory tract infection within the last 2 weeks (entry can be deferred)
Previous spontaneous pneumothorax without pleurodesis (bronchoscopy subgroup only)
Current smoker
Significant comorbidity including: Significant renal impairment (serum creatinine > 150 micromol/l) Moderate/severe CF liver disease (varices or sustained-ALT/AST>100IU/l) Significant coagulopathy (bronchoscopic group only)
Receiving 2nd line immunosuppressant drugs such as: methotrexate, cyclosporine, intravenous immunoglobulin preparations
Pregnant or breast feeding

Table 3.2-1: Inclusion and exclusion criteria for subjects participating in the Multidose Trial. *These were applicable to all subjects in the trial unless it is indicated specifically that it is only relevant to patients in either of the subgroups.*

3.2.5 Ethics and MHRA approval

This trial was approved by the Gene Therapy Advisory Committee (GTAC184/http://www.clinicaltrials.gov/ct2/show/NCT01621867) and the Medicines and Healthcare products Regulatory Agency. It was sponsored by Imperial College London and managed by the Imperial Clinical Trial Unit. Funding for the study was obtained from the NIHR/MRC EME board.

3.2.6 Study design

The study design and protocol were devised by the GTC's strategy group. This was a randomised, double blind placebo-controlled study designed to assess the clinical efficacy and tolerability, and gene expression of repeated doses of gene therapy. Randomisation was on a 1:1 basis, stratified for centre, age and FEV_1% predicted. An overview of the trial is illustrated in Figure 3.2-1

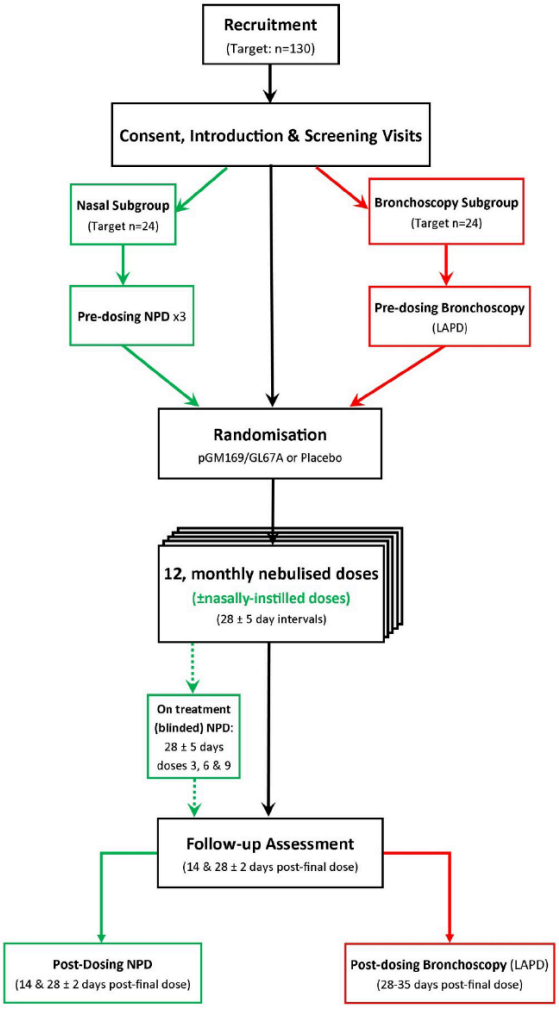


Figure 3.2-1: Overview of gene therapy Multidose Trial – nasal subgroup red, bronchoscopy sub group green. Reproduced with permission from Dr Michael Waller

All patients were recruited to receive 12 doses of nebulised gene therapy or placebo 28 (+/-5) days apart (lasting 48 weeks in total). Investigations were performed prior to the first dose, prior to select doses and again on completion on the trial (See Figure 3.2-2).

3.2.6.1 Mechanistic Subgroups

Patients seen at the RBH only, were invited to join either one or both subgroups; the nasal (n=24) and bronchoscopy (n=24), which were designed to look for evidence of gene expression. The randomisation of patients in the subgroups was 2:1 in favour of active treatment. The results of the nasal subgroup are being reviewed by my colleague Dr Michael Waller (CRF) and will not be covered in detail in this thesis, however mention will be included where appropriate.

Nasal subgroup:

In addition to the nebulised 'lung' dose of gene therapy, these patients received a 2ml nasal dose of gene therapy administered via a nasal spray. Participants underwent NPD tests at the regular time intervals for assessment of CFTR function, in addition to nasal brushings pre- and post-dosing and following completion of the trial to asses for the presence of CFTR DNA and vector specific mRNA.

Bronchoscopy subgroup:

Patients underwent two LAPD tests to assess bronchial epithelial CFTR function; these were performed pre-dosing and after completion of the trial. Brushings were taken on both occasions for measurement of CFTR DNA and vector specific mRNA levels. Endobronchial biopsies were performed to assess remodelling, lipid accumulation and inflammation.

3.2.6.2 Randomisation

Electronic randomisation was performed as soon as possible after the patient passed screening using the InForm system (a web-based data entry system provided by Imperial College). Relevant patient details were entered by a member of the trial team to generate a 4 digit randomisation number corresponding to a blinded randomised arm of the trial.

3.2.6.3 Study visits

An overview of the all the visits is provided in Table 3.2-3. All visits and investigations were performed within the timeframe as defined in the protocol and documented in Table 3.2-2. If this

was not possible, a discussion took place with the CI as to whether to adjust the timeframe. The timing of tests performed for safety monitoring was not adjusted.

Visit	Time frame	Description
Pre-dosing		
All subjects		
Introductory (optional)	N/A	Confirmation of subjects eligibility Consent obtained Baseline measurements
Screening (compulsory)	N/A	Re-confirmation of subjects eligibility Baseline measurements
Subgroups only		
Nasal	NPD: performed on any visit Brushings >7 days pre 1st dose	Confirmation of subjects eligibility NPD performed
Bronchoscopy	If pre-screening: > 7 days pre-screening If post-screening: > 7 days prior to 1st dose	Confirmation of subjects eligibility Bronchscopy performed
Dosing		
All subjects		
All dosing visits	Dose 1: <28 days from screening Other doses: 28 days +/- 5 days referenced to dose 1 (Extended to +/- 7 days in exceptional circumstances)	Clinical stability confirmed Dose given over 40 minutes with a minimum of 30 minutes observation before discharge
Doses 4/7/10		Specified additional tests performed
Subgroups only		
Nasal		Additional nasal dose of gene therapy administered
Follow up		
All subjects		
Follow up 1	14 days (+/-2) referenced to final dose	Post dosing measurements
Follow up 2	28 days (+/- 2) referenced to final dose	Post dosing measurements
Subgroups only		
Nasal	Performed at follow up 1 and 2 Nasal brushings after 2nd NPD	NPD performed
Bronchoscopy	27-36 days following the last dose ensuring after follow up	Bronchoscopy performed

Table 3.2-2: An overview of the study visits in the Multidose Trial.

3.2.6.4 Dosing protocol

Patients normally taking RhDNase (Pulmozyme) were asked to withhold treatment for 24 hours prior to each dose and for 24 hours after due to concerns over potential degradation of the *CFTR* genomic DNA.

20 minutes pre-dosing, patients received 200mcg of inhaled salbutamol via metered dose inhaler and spacer (unless there was a contraindication) to limit any potential bronchoconstriction induced by the hypotonic gene therapy formulation. Patients were provided with a nasal clip, and 5 ml doses of the product: pGM169/GL67A or placebo (0.9% saline) was delivered via an AeroEclipse breath-actuated nebuliser in specially designed negative pressure cubicles. The active product and the placebo were delivered in identical bottles to prevent un-blinding. Nebulisation was performed, with patients receiving 8 cycles consisting of 3 minutes 'on' and 2 minutes 'off' (40 minutes in total).

Nasal subgroup: A single spray to each nostril of the nasal dose was administered, by the patient, at the beginning and at the end of the 'off' period, a total of 6 times. The patient held their head forward by 45^{o}, placed the nasal spray nozzle in the nostril, and lifted the bottle to a 45^{o} angle. Whilst breathing in slowly through the nose, the subject depressed the bottle to generate a spray.

1g of paracetamol (dose adjusted for weight if <16 years old) was given immediately post dose and again 6 hours later, to mask any potential flu-like symptoms due to the active product which might cause un-blinding. Patients were observed for 30 minutes post-dose, either in the dosing cubicle or whilst wearing a facemask in the study bedroom, and reviewed by a doctor and pulse oximetry performed prior to discharge.

3.2.6.5 Adverse events and safety reporting

AEs were collected on all patients at every trial visit by one of the doctors. These were graded according to their severity and their relationship to the study drug. AEs were discussed cross-site to ensure consistency in grading and serious AEs were immediately reported to the trial sponsor and relevant bodies.

3.2.6.6 Long-term follow up

All patients, including those who had withdrawn after commencing dosing, continue to be followed up for a total of 2 years after finishing dosing, at their scheduled clinical appointments.

3.2.7 Outcome measures

The outcome measures included as part of the trial are listed in Table 3.2-3. This chapter describes in detail only those relevant and those utilised as outcome measures in the study described in Chapter 4.

Clinical efficacy	Safety
Relative change in lung function *	Clinical examination
Lung Clearance Index *	Transcutaneous oxygen saturations
Chest CT scan *	Serum inflammatory markers*
Exercise capacity (bike test)	Renal and hepatic function
Frequency of respiratory exacerbations Activity monitor	Endobronchial histology* (bronchoscopy subgroup only)
Serum calprotectin*	Immune response markers
Quality of life questionnaire	Gas transfer
Change in sputum weight*	
Sputum culture and inflammatory markers*	

Table 3.2-3: List of the outcome measures included as part of the MDT. All patients underwent all investigations at the time points illustrated in Figure 3.2-2 unless indicated for a subgroup only. Investigations documented with a * are of particular relevance to this thesis

Figure 3.2-2 provides a summary of all study investigations performed at individual time points.

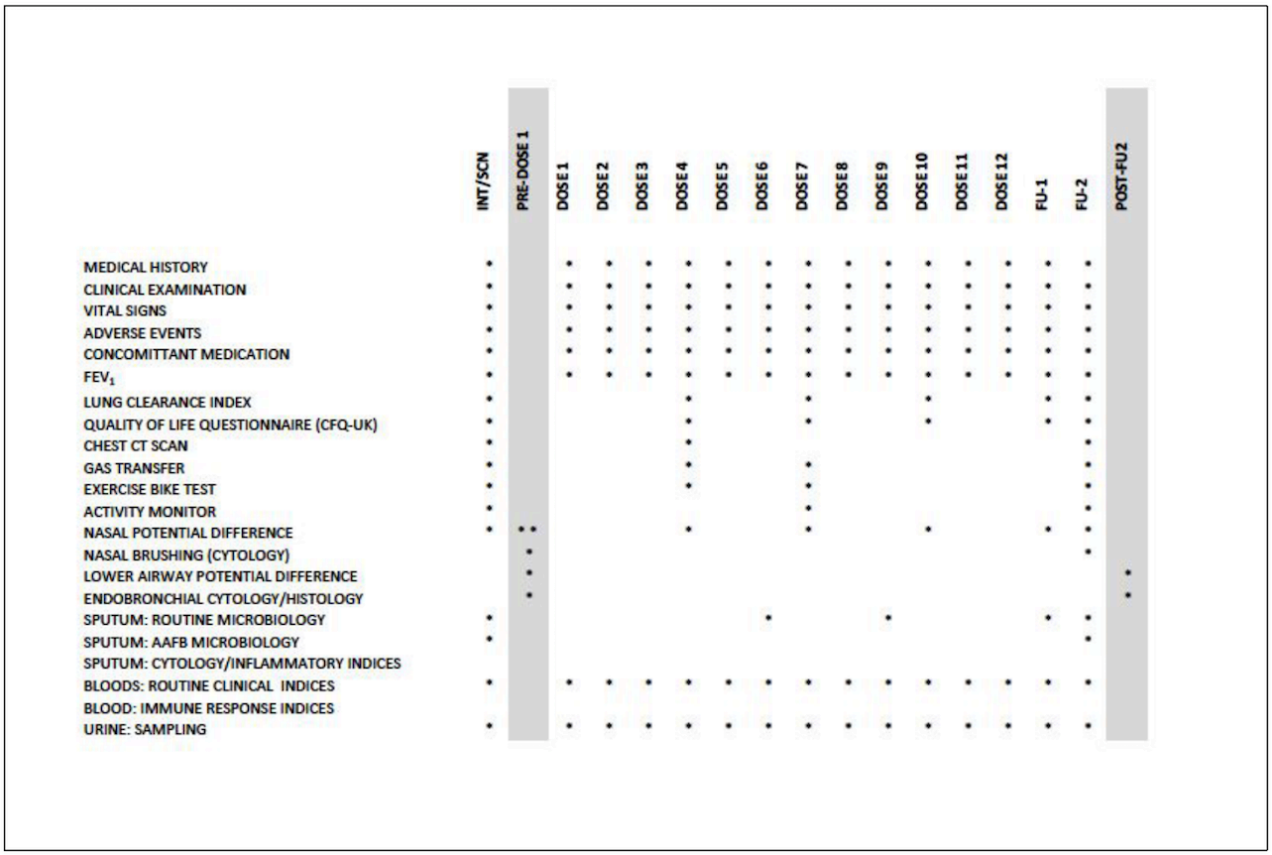


Figure 3.2-2: Summary of the study investigations at individual time points of the Multidose Trial. INT – Introductory visit; SCN- Screening visit; FU – Follow up visit; AAFB- Acid fast and Alcohol Fast Bacilli

3.2.7.1 Outcome measures of airway health

Spirometry (see Section 2.2.2)

FEV_1 % predicted was performed at screening to determine the subjects' eligibility for enrollment into the study, and on dosing visits to ensure that they met the fitness criteria to dose (Appendix B). Spirometry was always performed prior to dosing, and the values were converted to Stanojevic reference ranges [281].

Lung HRCT scan

All subjects underwent a total of 3 HRCT scans, pre and post dosing to assess efficacy of the treatment, and pre-dose 4, as a safety measure. HRCT scans were performed on a 64-channel multi detector scanner (Sensation 16 or 64; Siemens Medical Solutions, Erlangen, Germany). Females were required to have a negative urine pregnancy test on the day of the CT scan. 2 dosing proformas were used: a volumetric (inspiration) scan comprising thin sections (1.25mm) throughout the lung (used pre- and post-dosing), and an interspersed (expiration) scan with 1.25mm at 10mm intervals in order to reduce radiation exposure (used pre dose 4).

Scoring:

The scans were anonymised and scored by two independent radiologists who were blinded to subject identification and whether the CT was performed pre or post gene therapy dosing. Scans were analysed and graded as follows:

- Extent of bronchiectasis (scored 0-3 per lobe and the value averaged),
- Bronchiectasis severity and wall thickness (0-4 per lobe and the value averaged),
- Evidence of mucus plugging (scored 0-2 per lobe and the value averaged),
- Tree in bud pattern (scored 1-5 per lobe and the value averaged)
- Gas trapping (scored as a %) [282].

LCI (See Section 2.2.3)

Tests were performed on all patients at 2 separate occasions pre dosing, prior to doses 4, 7, 10 and at follow up 1 and 2.

To ensure quality control of data analysis at both clinical sites the following measures were put in place:

- A proportion (13.8%) of the LCIs were analysed at both sites, across a full range of patients with different LCI values, with tests taken from each time point to determine the inter-observer variability. The 95% limits of agreement were -0.04 to 0.04 LCI units.

- Due to the blinded nature of the trial, the majority of the traces were analysed following the completion of the trial, with the exception of those obtained pre dosing, and performed in batches related to the visit to prevent longitudinal un-blinding of the observer.

Inflammatory markers:

Inflammatory markers were assessed in the serum, the sputum from all subjects in the trial who were able to expectorate, and from endobronchial biopsies in subjects participating in the bronchoscopy subgroup.

Serum inflammatory markers

Blood samples were taken at each visit for both clinical and research assays. Samples were sent to the trial-site hospital laboratory for assessment of FBC, CRP, ESR, renal and liver function tests, and for the bronchoscopy subgroup only, coagulation prior to the procedure. At the time points indicated in Figure 3.2-2 blood was tested for anti-nuclear and anti-double stranded DNA antibodies in the Clinical Microbiology Laboratory, RBH and lymphocytes were removed from the whole blood and sent to the University of Pennsylvania for assessment of anti-CFTR T-cell responses. On one occasion blood was taken for the extraction of genomic DNA for CFTR genotyping and saved for polymorphism analysis of possible modifier genes; the latter has not been analysed to date.

Serum was frozen and stored for subsequent cytokine analysis described below. This was performed by the London GTC research technicians.

IL-6

Serum IL-6 was measured on the Beckman Access 2 immunoassay analyser, according to manufacturers guidelines (Beckman Coulter, High Wycombe, Buckinghamshire, UK)

Calprotectin:

The measurement of calprotectin in the serum samples was performed by an in-house calprotectin ELISA (intra-assay coefficient of variation = 5.6%; unpublished data). Calprotectin antibodies were gifted by Erling Sundrehagen, Oslo, Norway. Incubation was carried out in a damp box. Microtitre plates (Immulux HB, Q88 Dynex Technologies, Chantilly, VA, USA) were coated overnight at 4°C with 100 µl of anti-calprotectin (mouse anti-human) monoclonal antibody at 2.5 µg/ml in coating buffer (KPL/Insight Biotechnology). Plates were blocked with 1% bovine serum albumin (BSA) for 1 hour at 37 °C and washed four times with 0.05% Tween-20. Duplicate samples of 100 µl were added to the plate in 1:60,000 dilutions for sputum in PBS and 1:500 for serum (50% fetal calf serum in PBS). Positive controls of calprotectin (Immundiagnostic, Oxford Biosystems, Oxford, UK) were included. Human recombinant calprotectin (Cambridge Biosystems, Cambridge, UK) was used to produce 1.56–100 ng/ml standard curves. The samples: anti-calprotectin (chicken anti-human) polyclonal antibody at 1:5000 dilution; 100 µl of alkaline phosphatase-conjugated donkey anti-chicken IgG (Jackson ImmunoResearch, Suffolk, UK) at 1:5000
dilution were added to wells in three cycles of incubation at room temperature for 1 hour on a platform vibrator (450 rpm) followed by washings. Finally, 100 µl of BluePhos® Microwell Phosphatase Substrate System (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) was added to each well. Plates were incubated in the dark at 600 rpm until the blue colour developed, and reached an absorption of 620 nm on a Biotek plate reader. Sample concentrations were calculated using the five-parameter logistic (5PL) non-linear regression curve-fitting model [283].

Sputum inflammatory markers

Sputum samples were obtained where possible at the visits illustrated on Figure 3.2-2. The samples were collected over 24 hours into 50ml Falcon tubes and weighed. If the patient was unable to expectorate sputum spontaneously, induced sputum was performed with 7% hypertonic saline only at screening and at follow up. Freshly expectorated samples were stored on ice for a maximum of 2 hours and processed as described below. If still no sample was obtained, a cough swab was performed for microbiological culture. Samples were analysed for microscopy, culture and sensitivity (MC&S), total cell counts (counts/g), neutrophil count (%), concentration of IL-8

(ng/ml), concentration of calprotectin (μg/ml), extracellular DNA (μg/ml), quantification of lipid droplets and sputum solid content (%). Only the methodology of the outcome measures that were used in this thesis are described below.

Sputum processing

Whole sputum was transferred into a sterile Petri dish. The sputum plugs were identified and separated out using forceps into a pre-weighed falcon tube and treated with freshly prepared 0.1% dithiotreitol (Sigma-Aldrich, Dorset UK) in Dulbecco's phosphate buffered saline (D-PBS), at a ratio of 4ml:1g. Each aliquot was briefly vortexed, rotated for 15 minutes at 4^{o}C before dilution in an equal volume of D-PBS and filtered through a pre-moistened gauze to remove solid debris. The sample was centrifuged at 1200rpm for 10 minutes at 4^{o}C to produce the sputum sol phase, and the supernatant, which was transferred to cryovials for storage at -80^{o}C. The cell pellet was re-suspended in 0.9% D-PBS for analysis of total cell counts.

Cell counts

Cells were isolated from solubilised sputum as described above, and approximately 105 sputum cells were cytospun (5 minutes at 500 rpm) onto cytoslides (Thermo Shandon Ltd, Cheshire, UK) (six slides per subject). Slides were air dried and fixed for 10 minutes in 4% formalin (Sigma-Aldrich, St Louis, M0, USA) and subsequently stored at – 20 °C until further use. For total and differential cells counts, cells were fixed for 10 minutes in cold methanol (Fisher Scientific, Loughborough, UK), dried and stained with May–Grunwald– Giemsa (MGG quick stain; Bio-Optica, Milan, Italy) using routine histological procedures and neutrophils, lymphocytes, macrophages and eosinophils quantified. Cells were counted on random fields until 300 neutrophils were counted, and adjusted for sample volume to provide cells/g sputum.

IL-8

The measurement of IL-8 in the sputum samples was performed using IL-8 Human ELISA and is described in Section 2.2.5.1.

Calprotectin

The measurement of calprotectin in the sputum samples was performed using the same in-house ELISA as described above for measurement of serum calprotectin.

Endobronchial biopsies:

Two endobronchial biopsies were taken per patient per bronchoscopy, obtained from approximately the 5th generation, using one set of disposable cup forceps (Olympus, FB-231). One biopsy was frozen in 2-methylbutane-cooled liquid nitrogen at –196 °C and the other immediately fixed in 10% formal saline.

The formalin-fixed samples were processed using a Tissue Tek Sakura VIP processor and embedded in paraffin wax blocks. 3 micron sections were then cut from these blocks for haematoxylin and eosin (H&E) staining. For H&E staining, slides were thawed at room temperature for 30 minutes, and then placed in Harris' s haematoxylin for 1 minute. Subsequently, they were differentiated in acid alcohol for 5 seconds and washed in tap water for 5 minutes. They were then counterstained with eosin for 30 seconds and cover-slipped using an aqueous mountant. These slides were scored independently by two blinded pathologists using a semi-quantitative scoring system from 0 to 6 for goblet cell hyperplasia, basement membrane thickening, presence of chronic inflammatory cells (lymphocytes and plasma cells), neutrophils, eosinophils and seromucinous gland hyperplasia, except for seromucinous gland hyperplasia which was scored as absent (0)/present (1).

The frozen biopsy sample was later analysed for the presence of lipid-laden macrophages using a semi-quantitative scoring system. This was required as a safety outcome to ensure that excessive lipids from the vector were not accumulating in the airway.

3.2.7.2 Methodology for outcome measures of gene expression

CFTR DNA and mRNA

Epithelial cell brushings

Sample collection

Nasal brushings were performed in subjects in the nasal subgroup following their final NPD measurements both pre-dosing, and on completion of the trial. Samples were taken from both nostrils (described in Section 2.2.4)

Lower airway brushings were taken from patients in the bronchoscopy subgroup following LAPD measurements. 10 bronchial samples were taken per patient per bronchoscopy using disposable cytology brushes (Olympus, BC-202D-5010). At the sampling site, the bronchial wall was brushed, and the bronchoscope removed from patient. The clinician performing bronchoscopy held the bronchoscope and sheath of cytology brush and wire brush beyond distal tip of scope. The wire brush was protruded further than the plastic sheath. A designated person held the wire (not the plastic casing) and cut the wire so they are left holding the brushing sample, taking care not to touch the brushings. The wire and sheath were retracted and removed through the bronchoscope. The site of the bronchial brushings was recorded and the procedure repeated as required.

Processing samples

Upper and lower airway samples were separately pooled and assessed for cell count and the presence of pGM169-specific and endogenous *CFTR* DNA and mRNA. This was performed using TaqMan (Life Technologoes, Paisley, UK) real time quantification PCR instruments and supplies [284]. These assays were run by Consortium researchers in Oxford according to the relevant SOP developed in that lab. Data were counted as percentage of pGM169-specific to endogenous CFTR numbers. If pGM169 was detected but below the linear detection range, or the quantification of endogenous CFTR signal was negative; the sample was scored as Positive But Not Quantifiable (PBNQ). Samples that were negative for endogenous CFTR were recorded as Not Determined (ND), and those negative for pGM169 but positive for endogenous CFTR were valued as zero. Results are presented as differences between post- and pre-treatment.

NPD measurements (described in Section 2.2.8.1)

Measurements were performed at the times illustrated in Figure 3.2-2; 3 pre-dosing, during, and twice post-dosing, most commonly coinciding with the follow up visits. As all NPD measurements were performed by myself or my colleague, it was necessary that the traces performed *during* the dosing period were not visible to the operator or the patient in case clear variations from an ‘expected CF trace’ led to un-blinding. This was achieved by a method designed for this trial in which an external member of the team with no clinical contact with the patients was trained to monitor the trace in real-time. They informed the operator when stability was obtained during the Ringers and Amiloride phases and if there was a sudden loss of contact between the catheter and the nasal epithelium. The final 2 stages were timed for 5 minutes each.

The validity of this 2-operator (blinded) technique was reviewed by Dr Waller, who showed that it did not impair the quality of the NPDs [196].

3.3 Statistical analysis

A total patient number of 120 was chosen to provide 90% power to detect a 6% difference between groups calculated as the mean change from baseline at a 2-sided 5% significance level.

Statistical analyses were performed according to a predefined plan devised by the trial statistician (Gordon Murray) and approved by the Trial Steering Committee. Statistics were performed using Prism (Version 5.0c for Mac OSX) and/or IBM SPSS Statistics (Version 22.0). Outcome between groups was compared with ANCOVA model, stratified for the following factors; gender, study site (London or Edinburgh), baseline FEV_1 and whether the subject was in a subgroup. A post hoc analysis was performed on all major endpoints, comparing the results between 70-90% (high) and 50-70% (low) starting FEV_1 predicted.

The results are displayed as adjusted mean differences and 95% confidence intervals (CIs). Bronchial and nasal biomarkers were assessed with a Mann-Whitney *U* test. The null hypothesis was rejected at $p<0.05$.

3.4 Results

3.4.1 Patient data

Patients were recruited between June 12, 2012 and June 24, 2013. 140 subjects were randomised into the trial to receive either the active product; n=78 or the placebo; n=62. 4 withdrew without receiving a dose leaving 136 patients (the intention to treat population); active n=76 and placebo, n=60. 116 subjects received ≥ 9 doses, active n=62 and placebo n=54. The reasons for discontinuing the trial were similar in both groups. Figure 3.4-1 illustrates patient numbers from enrolment to completion of the study.

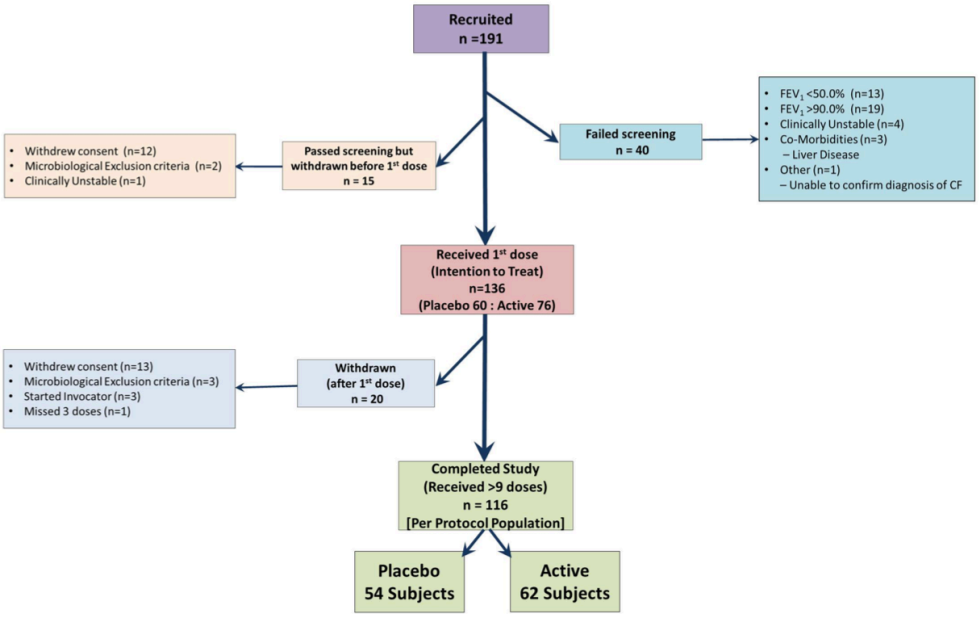


Figure 3.4-1: Consort diagram illustrating patient numbers from enrolment to completion of the study.

3.4.2 Subject characteristics

Baseline characteristics were similar between the two groups as illustrated in Table 3.4-1. The mean (SD) age of subjects in the active group was 26 (13) compared to 23.6 (10.8) in the placebo. The mean (SD) FEV_1 % predicted in the active group was 69.0 (9.9) compared to 69.9 (11.1)%.

	Placebo	Active
Subjects	54	62
Age (years) mean (SD)	26.0 (13.0)	23.6 (10.8)
<18	17 (31%)	23 (37%)
>18	37 (69%)	39 (63%)
Sex		
Female	25 (46%)	31 (50%)
Male	29 (54%)	31 (50%)
Centre distribution number		
Edinburgh	24 (44%)	22 (35%)
London	30 (56%)	40 (65%)
Height (cm) mean (SD)	165.0 (10.6)	163.6 (10.9)
Weight (kg) mean (SD)	61.6 (15.6)	61.0 (15.7)
FEV_1 (% predicted) mean (SD)	69.0 (9.9)	69.9 (11.1)
Body-mass index (kg/m2) mean (SD)	22.4 (4.4)	22.4 (4.5)
Mutation class		
F508del/F508del	26 (48%)	31 (50%)
F508del/class 1-6	22 (41%)	23 (37%)
Not F508del/class 1	1 (2%)	3 (5%)
Heterozygous/ homozygous class 3-6	2 (4%)	2 (3%)
F508del/ Unknown class	3 (6%)	3 (5%)

Table 3.4-1: Baseline and demographic characteristics of subjects in the Multidose Trial

3.4.3 Primary outcome measure

Out of 116 subjects fulfilling predefined per protocol criteria, pre and post treatment measurements of % predicted FEV_1 were available for 114 patients (active 60, placebo 54). Of the two that were not available for follow up, 1 subject was excluded due to clinical deterioration and the other withdrew due to time commitments. At 12 months, a significant ANCOVA-adjusted treatment effect was demonstrated in the active group compared to the placebo group; 3.7% (95% CI 0.1- -7.3), p=0.046. The relative changes demonstrated between the 2 groups were -0.4% (95% CI -2.8 - 21) in the active group compared to -4.0% (95% CI -6.6 - -1.4) in placebo. The treatment effect was evident from 1 month onwards, as seen in Figure 3.4-2, which illustrates the time course of FEV_1 % predicted values in patients in response to both active and placebo.

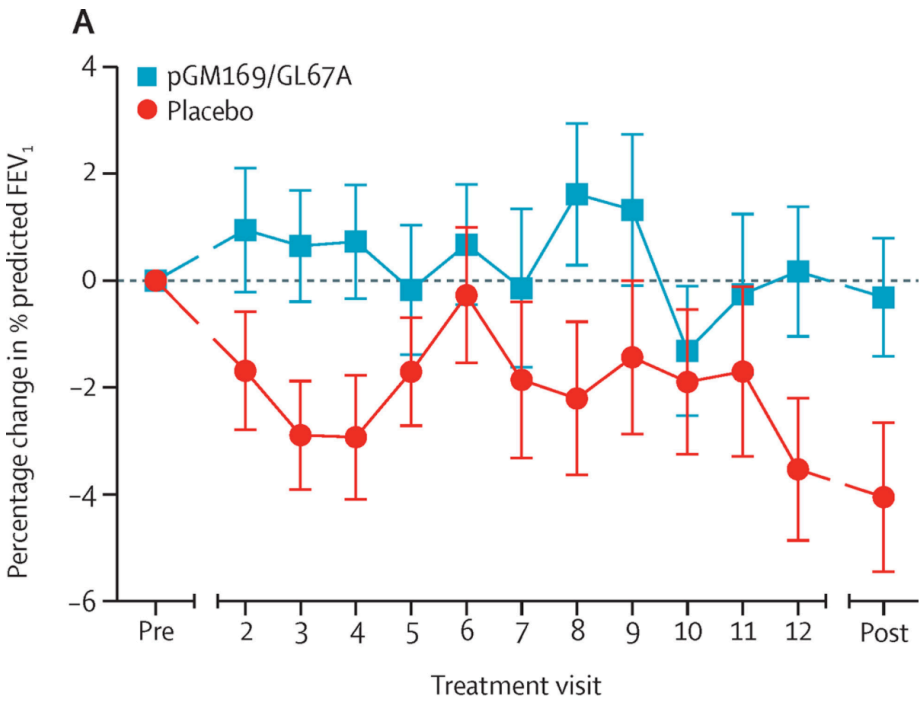


Figure 3.4-2: Time-course of the change in FEV_1 % predicted value in response to placebo or gene therapy (pGM169/GL67A). *Error bars show the standard error of the mean*

To determine if a responder subgroup existed the patients were divided into pre-defined groups and analysed according to sex, age, CFTR mutation (F508del homozygous vs other), chronic infection with *PA,* severity of disease as according to starting FEV_1%, CT findings, concurrent medication and AEs considered to be associated with trial medication. This revealed a trend, though not a significant difference between subjects with more severe disease (FEV_1 49.6-69.2%) demonstrating a greater response than those with less severe disease (69.6-89.9%); +6.4% (95% CI 0.8 - 12.1%) compared to +0.2% (-4.6 - 4.9) (p=0.065). This is illustrated in Figure 3.4-3.

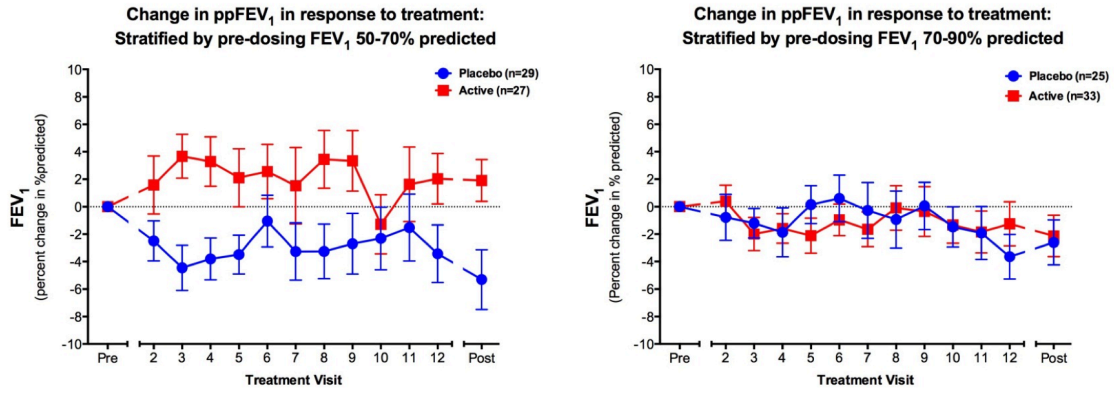


Figure 3.4-3: Primary outcome stratified by baseline FEV_1. Time course of the primary outcome response stratified by baseline FEV_1 at trial entry. (a) Severe group, baseline FEV_1 49.6–69.2% predicted; and (b) milder group, baseline FEV_1 69.6–89.9%, showing a trend towards a larger effect in the severe patents (P=0.065). *Error bars indicate standard error of the mean.*

Following the observation that baseline FEV_1 appeared to have an influence on the magnitude of the treatment effect based on the primary outcome, other outcome measures were analysed for this effect. Many of the assays reflected the effect that had been seen for the primary outcome, demonstrating that the more severe group of patients showed approximately double the treatment effect compared with the values in the whole, unstratified group. This resulted in an absolute improvement (vs. stabilisation) in many of the assays. Of note, in those with less severe FEV_1 at trial entry, biomarkers associated with smaller airways, in particular LCI, still showed a trend towards improvement, favouring the active group. These benefits seen in the severe subgroup were not related to an increased number of antibiotic courses during the trial, as the number of courses taken was comparable in both groups of disease severity.

3.4.4 Secondary outcome measures results

A significant treatment effect was also demonstrated for FVC (p=0.03), and change in extent of gas trapping seen on HRCT scans (p=0.048) but not for other measures of lung function including LCI, imaging, or quality of life assessments. Whilst the study was not powered to detect differences in other outcome measures, a trend was seen across the parameters taken together in favour of active treatment over placebo, which did not reach statistical significance. These are illustrated in Figure 3.4-4, which summarises the primary and secondary trial outcomes, in response to placebo and active treatment.

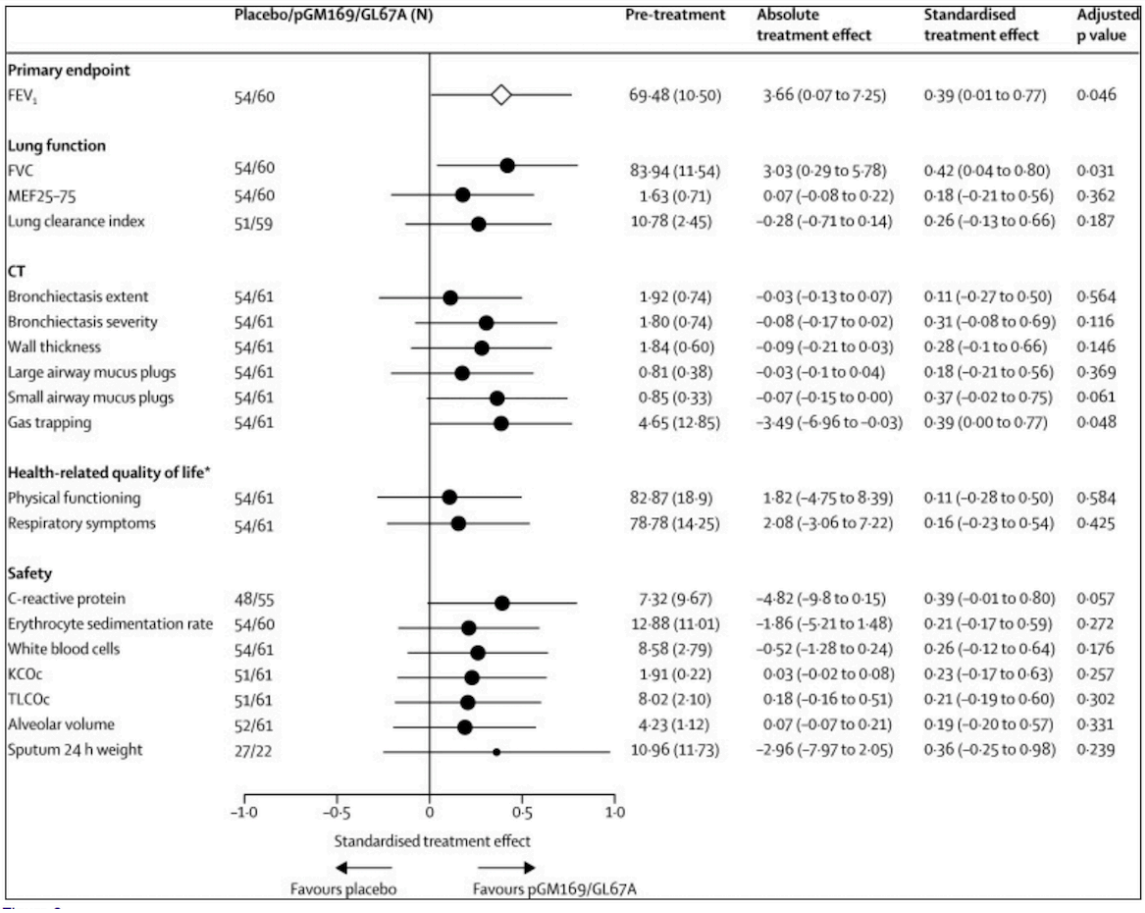


Figure 3.4-4: Forest plot showing secondary outcome responses to placebo or the active product (pGM169/GL67A). *Data are mean (SD) or mean (95% CI) unless otherwise indicated.*

There was no difference between those in the active and placebo groups between the numbers of oral antibiotic course received (p=0.3) or i.v courses (p=0.33), which was used as a surrogate measure of pulmonary exacerbations, although the trial was not powered to demonstrate it.

3.4.5 Adverse events

No statistical different was found between the total numbers of adverse events recorded between the placebo and treatment groups (p=0.68) with a mean of 19.1 per patient throughout the trial in the placebo group compared to 21.1 in the active group.

3.4.6 Inflammatory markers

3.4.6.1 Serum:

Serum samples were collected in all patients. No differences were detected in the level of inflammatory markers in the serum between the active and placebo group. A comparison of the changes in serum CRP/ ESR and WCC are illustrated in Figure 3.4-4 and changes in other markers of inflammation are illustrated in Figure 3.4-5.

3.4.6.2 Sputum:

Out of the expected 232 sputum samples, 169 (63%) were not available because patients did not expectorate sufficient sputum for processing. No differences were detected in the level of inflammatory markers in the sputum between the active and placebo group (Figure 3.4-5)

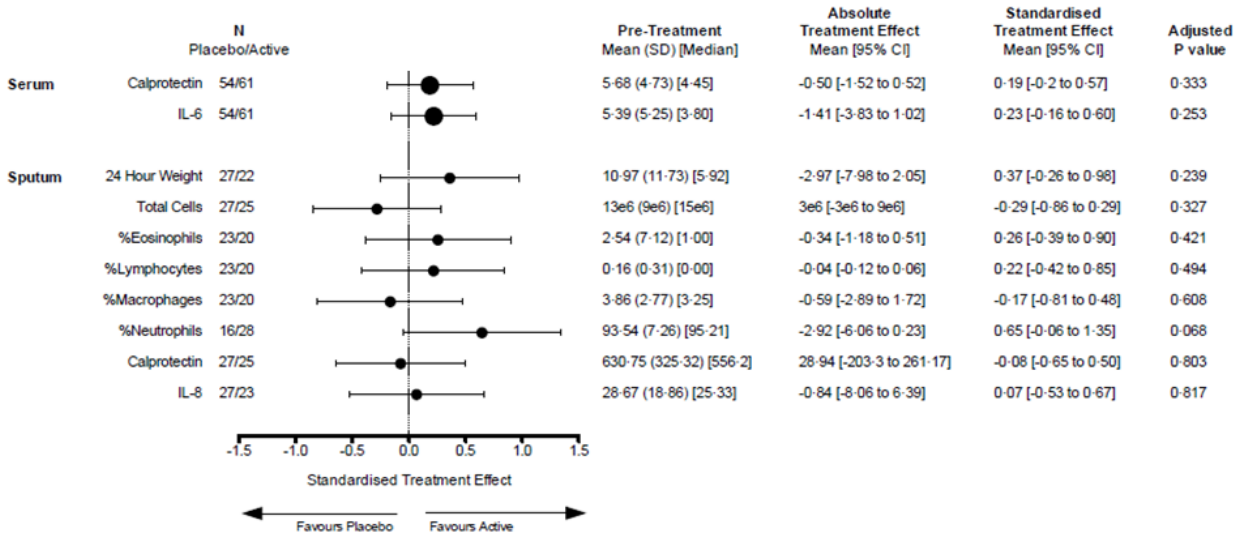


Figure 3.4-5: Forest plot showing the change in sputum and serum inflammatory mediators to placebo or the active product (pGM169/GL67A)

There were no clinically relevant changes in sputum microbiology including: *Aspergillus fumigatus, Mycobacteria, HI, PA* and *SA*

3.4.6.3 Endobronchial biopsies:

Out of the 50 possible biopsies, 39 (78%) were analysed. The remaining 11 biopsies were not analysable because of poor sample quality (n = 10) or because the patient withdrew from the trial (n=1), as illustrated in Figure 3.4-6, with no difference between the active and placebo groups.

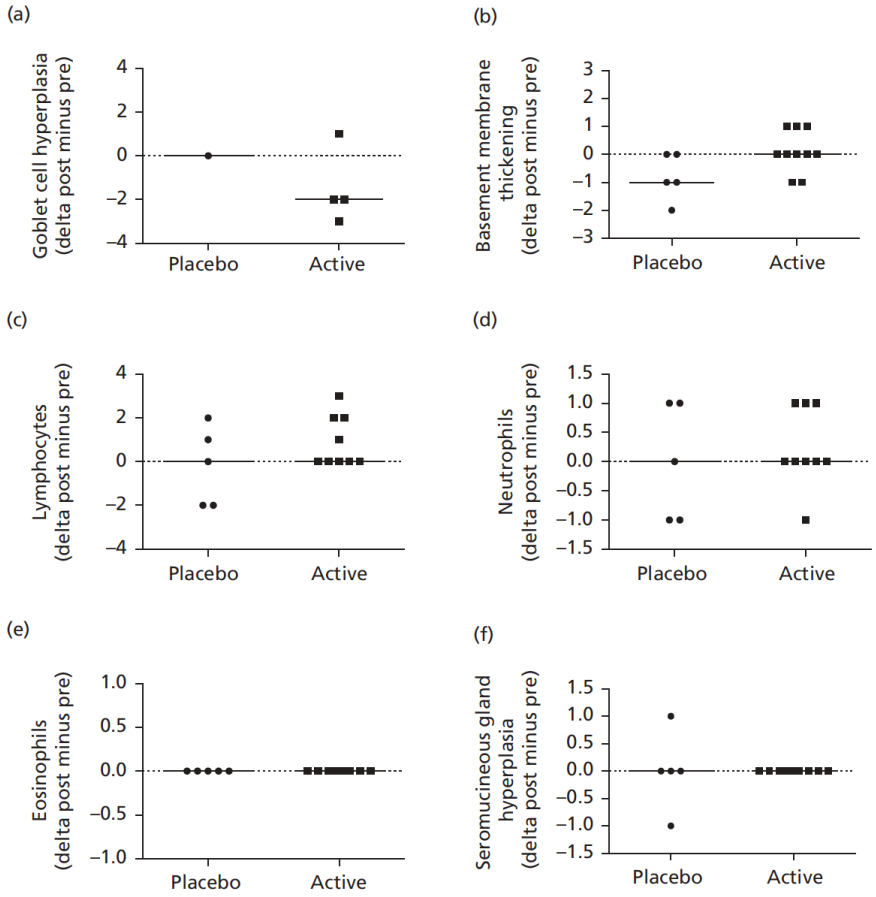


Figure 3.4-6: Histological assessment of bronchial biopsies. All data are expressed as the change from post to pretreatment. A positive number indicates an increased score following treatment. Dots represent scores for individual biopsies. The horizontal bar indicates the median. a) goblet cell hyperplasia; b) basement membrane thickening; c) numbers of lymphocytes; d) numbers of neutrophils; e) numbers of eosinophils; and f) seromucinous gland hyperplasia.

3.4.7 Outcome markers of gene expression

3.4.7.1 Molecular CFTR results

As expected, in the nasal group vector specific DNA was detected in all 15 of the patients who had received the product. There was evidence of cross contamination in some samples, however significantly more was detected in the active group compared to placebo. In the lower airway, the level of vector specific DNA was significantly increased in the patients who had received the active product (12/14) compared to none in the patients in the placebo group (0/7) (Figure 3.4-7) (p=0.001). This demonstrated that the product made contact with both the nasal and bronchial epithelium and was still present at approximately 28 days post-dose. The specific mRNA levels were below the limits for detection for both groups in both the upper and lower airway.

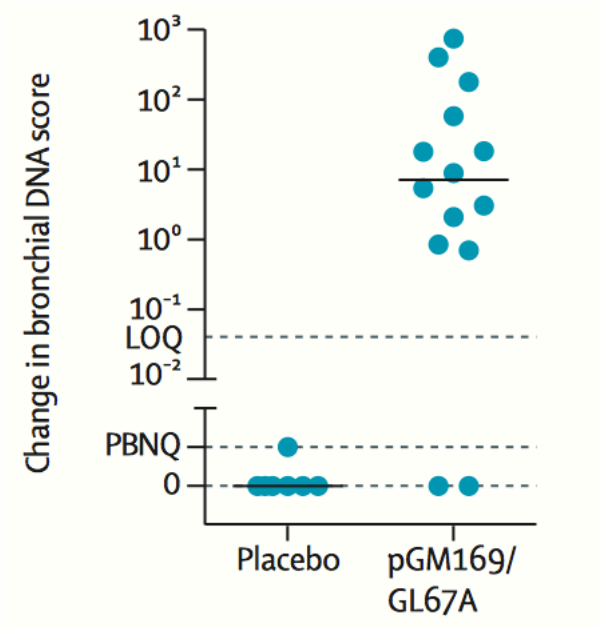


Figure 3.4-7: Comparison of change in vector specific DNA between active and placebo group post dosing. *Positive But Not Quantifiable = PBNQ, LOQ = limits of quantification, pGM169/GL67A = active product. The horizontal line illustrates the median*

3.4.7.2 Nasal subgroup - NPD measurements

24 subjects were in the nasal subgroup. 1 patient was excluded prior to the final analysis as the pre-dosing traces were post-hoc considered to demonstrate a total chloride secretory response ≥ 5 mV. Overall, 75/106 (70.8%) of ZCl, and 70/106 (66.0%) of ZCl/Iso recordings were considered interpretable. No signifiant changes were demonstrated in either the baseline values, amiloride or chloride secretory responses in either group, illustrated in Figure 3.4-8. However, as in previous trials a number of patients in the active group showed changes in chloride values towards non-CF values.

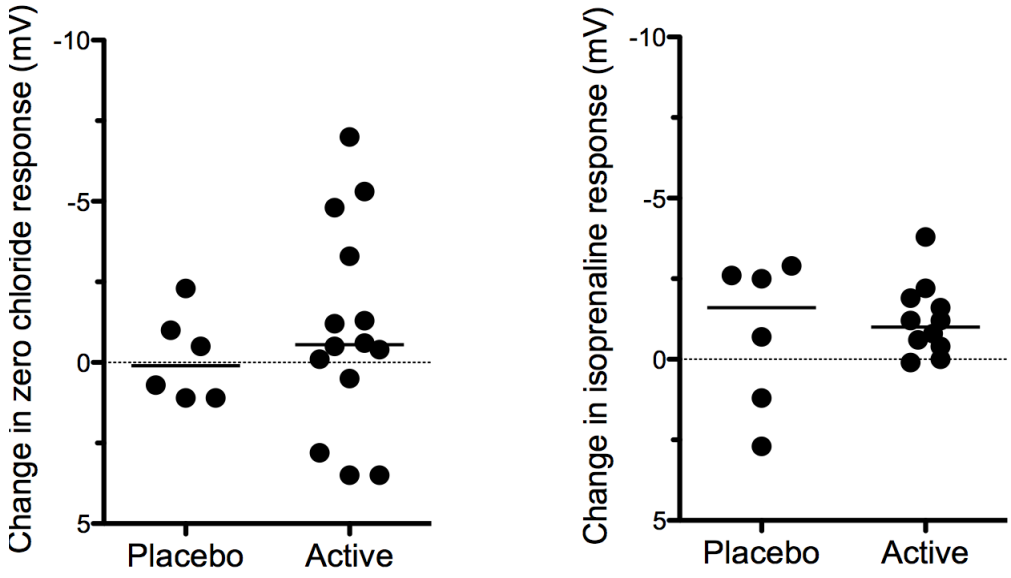


Figure 3.4-8: Comparison between change in chloride secretion in NPD between active and placebo groups post dosing. a) change in voltage in response to perfusion of ZCl solution and b) change in voltage in response to perfusion of ZCl/Iso solution. *The horizontal lines indicate the median.*

3.5 Discussion of primary and secondary outcome measures

The gene therapy Multidose Trial is the first trial designed to assess the clinical efficacy, safety and tolerability and gene expression, following repeat doses of non-viral CFTR gene therapy in patients with CF. The primary outcome demonstrated a statistically significant treatment effect with relative change in FEV_1% predicted in the active group 3.7% higher than in the placebo group (p=0.046) (relative changes of -0.4% and -4.0% respectively). Changes in secondary outcome measures showed a trend towards supporting this finding; however only differences in FVC and the percentage change in gas trapping detected by HRCT reached statistical significance. No clinically important AEs attributable to the active treatment were seen. The results of the mechanistic outcome measures are discussed in the next section along with the results from the LAPD measurements.

These results are encouraging, however the treatment effect was largely due to decreased FEV_1 in the placebo group associated with stabilisation of lung function in the active group. A reduction in FEV_1 in a placebo group has been demonstrated in other clinical trials in CF but here was higher

than that recorded in the CF registry of 1-2%/year [129, 285, 286]. The strict entry criteria for entry into the trial may have resulted in patients being at optimal health, and subsequently normalising over the forthcoming 12 months. In addition, the CF registry may underestimate the rate of annual decline as annual patient data is generally collected at a time of patient stability, thus not reflecting real-life variations.

The largest improvement in FEV_1 was seen in patients with more severe disease. This is likely to be due to patients with more severe disease having more obstructed distal (small airways) and the product predominantly being delivered to the proximal (larger airways) compared to those with milder disease. Preliminary studies, which were performed to assess drug deposition in the airway using similar sized particles to the gene therapy product, demonstrated almost twice the deposition in the larger airways in patients with more severe disease, thus supporting this theory. Correspondingly a trend towards a greater improvement in LCI (distal airway) was seen in those with mild disease compared to severe.

Whilst this trial demonstrated stabilisation of FEV_1, gene therapy is unlikely to be included in patient's clinical care due to already high treatment burden on patients and large treatment cost in CF. Further work in this field involves to try and improve the efficacy of gene transfer. Using the non-viral vector this involves studies reviewing the frequency of dosing, and potentially assessing the effect of a combination of gene therapy with a CFTR potentiator. The GTC is currently working on a 'wave 2' project which focuses on the development of a novel viral vector. This project is looking at lentiviral vectors as that they appear to evade the immune system, allowing a prolonged expression profile, however they are inefficient at transducing airway epithelial cells. The consortium have pseudotyped the lentivirus with envelope proteins (F and HN) derived from the Sendai virus (SIV) in order to increase its ability to transfect airway cells, showing that the F/HN-pseudotyped simian immunodeficiency viral vector (F/HN-SIV) can be delivered repeatedly and safely to murine lower airways without loss of expression, and that it also transduces human air-liquid interface (ALI) cultures generating stable, long term gene expression [287]. Extensive safety testing must be carried out prior to human clinical trials, as therapy that integrates the imported gene into the host cell DNA risks insertional mutagenesis and possible activation of a proto-oncogene, as seen in patients who received gene therapy for Severe Combined Immune Deficiency (SCID), who subsequently developed leukaemia [288].

This chapter has set the scene for Chapter 4, which focuses on the bronchoscopy subgroup and the LAPD outcomes. This subgroup provided the opportunity to explore the relationship between CFTR ion function in the lower airway with measures of disease severity and inflammation at baseline and the changes in these parameters following gene therapy.

4 Relationship between lower airway potential difference measurements and airway disease

4.1 Introduction

LAPD measurements were chosen as an outcome measure in the Multidose Trial to evaluate CFTR function, and provide proof of successful gene transfer. LAPD measurements are infrequently performed, and to date, no studies have compared these measurements to markers of disease severity. As LAPD measures the ion channel function of CFTR in bronchial epithelium, the direct site of disease pathology, these measurements are likely to correlate with markers of airway disease.

The hypothesis of this study stated that increased expression of CFTR, as measured by LAPD, would provide a protective effect on disease progression and would have an inverse association with markers of airway disease. The secondary hypothesis was that improvements in airway PD measurements following repeat dosing of CFTR gene therapy would directly correlate with an improvement in clinical markers of airway disease.

Evaluating the relationship between LAPD measurements and markers of airway disease

4.2 Study aims:

- To compare LAPD measurements with functional and structural markers of airway health in patients with CF

- To establish the relationship between changes in LAPD and those in clinically relevant markers after repeated dosing with CFTR-gene therapy

Objectives:

- To measure LAPD in patients with CF prior to starting gene therapy, and compare the parameters derived from this with the baseline measurements of inflammation which were collected in these patients as part of the MDT

- To measure LAPD in patients with CF after repeat doses of lipid-mediated gene therapy and compare the changes in these parameters with the changes in inflammatory markers

4.3 Study design

Data from patients taking part in the bronchoscopy subgroup of the gene therapy Multidose Trial were included in this study (inclusion criteria are listed in Table 3.2-1). Patients underwent a bronchoscopy prior to commencing gene therapy during which LAPD measurements were performed, and again on completion of the trial after the follow up measurements had been obtained (27-32 days post last dose). LAPD was used as a functional assessment of CFTR. Other measurements analysed are listed below, and were obtained at the time points as described in Chapter 3.

Measurement of CFTR

- Functional assessment
    - LAPD measurements
- Molecular assessment
    - Bronchial brushings for detection of vector specific CFTR DNA and mRNA

Measurements of airway disease and inflammation

- Spirometry
- LCI
- Serum markers of inflammation
    - CRP/WCC/ESR/Calprotectin/ IL-6
- Sputum markers of inflammation
    - Total cell count/ neutrophils/ calprotectin/ IL-8
- Endobronchial inflammation (assessed from endobronchial biopsy)

4.4 Methods

The methodology for obtaining the outcome measures are described in Section 3.2.7. LAPD measurements were performed as described in Section 2.2.8.2.

4.4.1 LAPD analysis

Professor Davies and myself analysed the basal values at the carina and in the distal bronchi. The chloride secretion, measured by the change voltage following perfusion of ZCl/Iso solution, was analysed by Professor Alton. As it is currently unknown whether the mean voltage measured, or the most negative voltage measured, is the most clinically useful parameter, both of these values were documented for the basal measurements at the carina, basal measurements in the distal airway and the values for chloride secretion.

4.4.1.1 Basal measurements

Each of the group of traces measured at the carina, and the separate traces measured distally (1-3); pre- and post- gene therapy, received an individual random number (generated with Excel random number generator). Every trace was analysed individually, with the operator blinded to the subject, treatment group, the timing of the trace, and to all other traces from that subject.

Analysis of the trace was performed in conjunction with the timings that were documented on the pro-forma at the time of measurement. Typical traces of the basal measurements obtained at the carina, and the distal basal measurements and the response to ZCl/Iso solution are illustrated in Figure 4.4-1 and Figure 4.4-2 respectively.

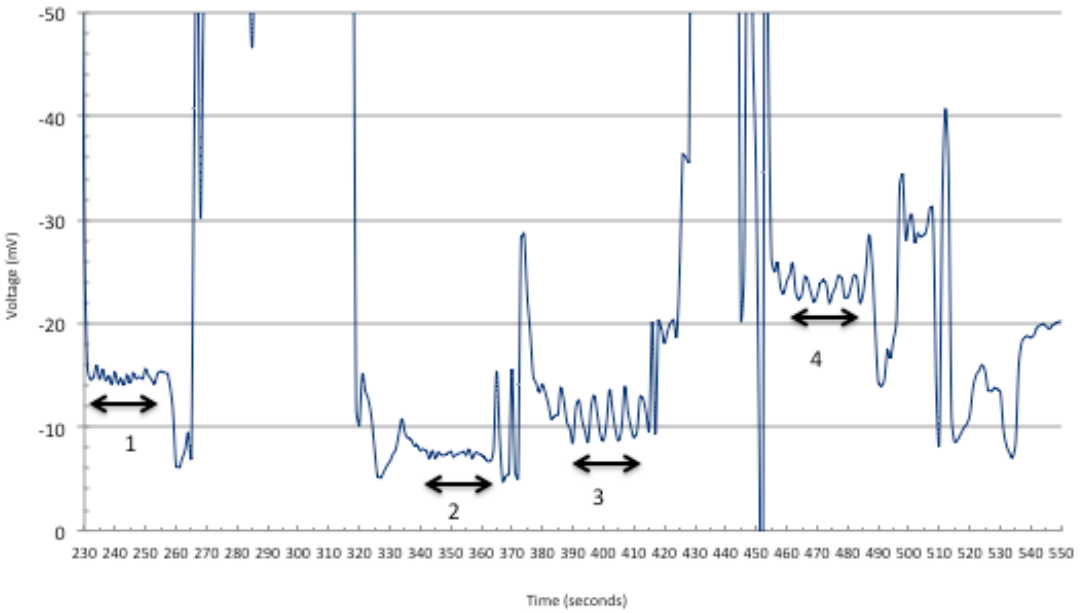


Figure 4.4-1: Typical traces of basal LAPD measurements at the carina – the 4 different locations (either anterior/ posterior/ right and left, measured in any order) are marked with the arrows 1-4 with the flat 'stable' trace representing the measurement. The final value is the mean voltage across this time.

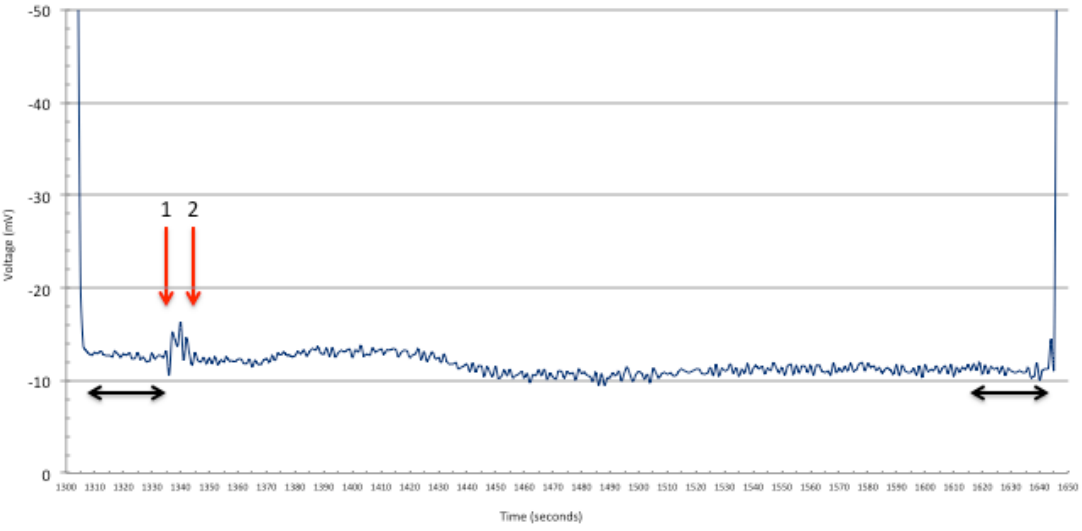


Figure 4.4-2: Typical LAPD trace in the distal airway of a CF subject – the 1st black arrow indicates the distal basal measurement (a mean of the values over 20 seconds of stability), the 1st red arrow where the Ringers solution was stopped, and the 2nd red arrow when perfusion of the ZCl/Iso solution was commenced. The 2nd black arrow indicates the final measurement after 300 seconds of ZCl/Iso perfusion (again a mean of the values over 20 seconds of stability. This shows no change in the voltage of the trace, consistent with absent chloride secretion, characteristic of CF.

4.4.1.2 Carina

Using the time and location documented on the pro-forma, the parts of the trace corresponding with the anterior/ posterior/ right lateral and left lateral tracheal measurements were identified. The stable period for each location was identified on the graph, and the mean value over this period taken as the final value. Measurements were unacceptable if the period of stability was <10 seconds or the trace was unsteady (rising or falling), illustrated in Figure 4.4-3.

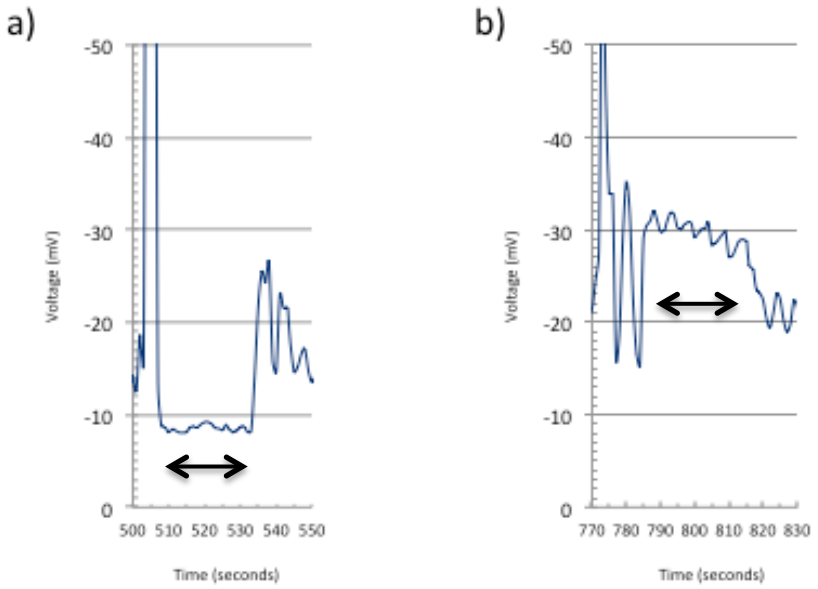


Figure 4.4-3: Examples of analysis of measurements performed at the carina. a) an acceptable measurement with the black arrow indicating the period of stability over which the values were averaged b) a measurement that was too unsteady to analyse, the black arrow illustrates this period, in which there is a downwards trend in the trace

4.4.1.3 Distal airway

Basal value: This was taken as the period of trace stability during the Ringers phase prior to changing the solution. The mean value for approximately 20 seconds preceding the transition was taken as the basal value. Measurements were unacceptable if the period of stability was <10 secs, the trace was thought to be rising or falling, or the trace was lost (Figure 4.4-4). There were no traces where the distal basal period was too short or unsteady, however on occasions the trace was lost and the measurements were subsequently abandoned.

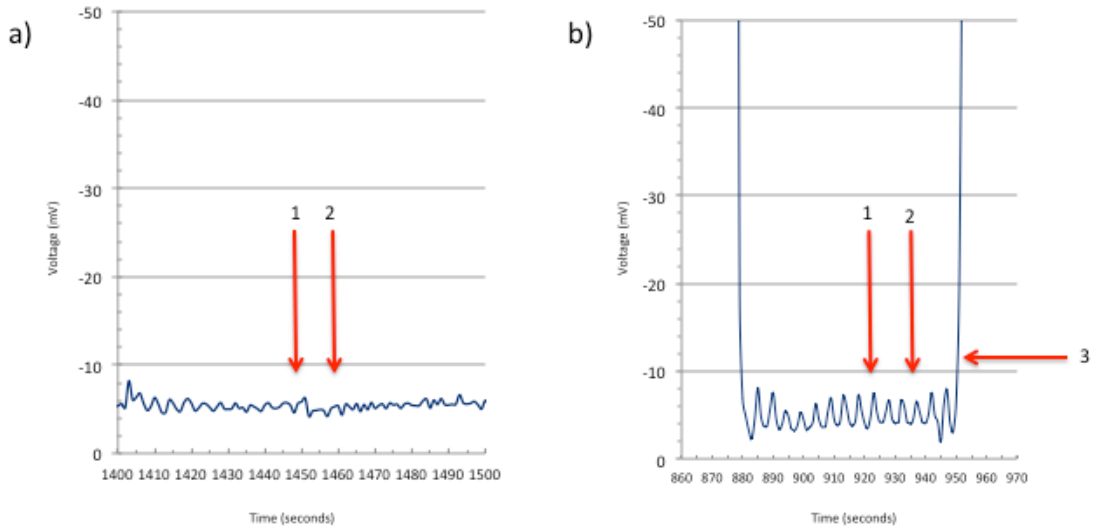


Figure 4.4-4: Examples of analysis of distal basal measurements performed at the carina. Arrow 1 indicates where Ringers solution was discontinued 2, where ZCl/Iso was started. a) illustrates an acceptable measurement and b) where the trace was lost (marked with the red arrow 3) and the measurement was abandoned

4.4.1.4 Chloride secretion

The bronchial potential recordings (1-3) from the same patient at the same time point were pre-grouped and the group assigned a random number. Traces were analysed as a group with the operator blinded to the subject, treatment group and whether the traces were performed pre- or post- dosing. The traces were printed and assessed visually. The values were obtained by meaning the data points in the stable segment of the trace.

The time for ZCl solution to reach the epithelium after commencing the perfusion of the fluid through the circuit was 50 seconds. Following perfusion of the ZCl/Iso solution the voltage either remained similar, became more negative (upwards), became more positive (downwards), or displayed both upward and downward deflection phases. Traces were initially assessed for acceptability and were accepted only if:

The time of any change in the trace occurred >50 seconds after commencing the ZCl/Iso solution (allowing the solution to pass through the catheter) and <80 secs, allowing approximately 30 seconds for drug interaction. If a deflection occurred outside these time points it was thought to be

more likely to as a result of external factors e.g. electrical interference secondary to the circuit change, dislodgement of mucus or localised debris, or electrode drift.

Traces were excluded if:

- The trace at 50-80 seconds was unstable
- The trace deflected both upwards and downwards (depolarisation and hyperpolarisation)

Examples of these are illustrated in Figure 4.4-5:

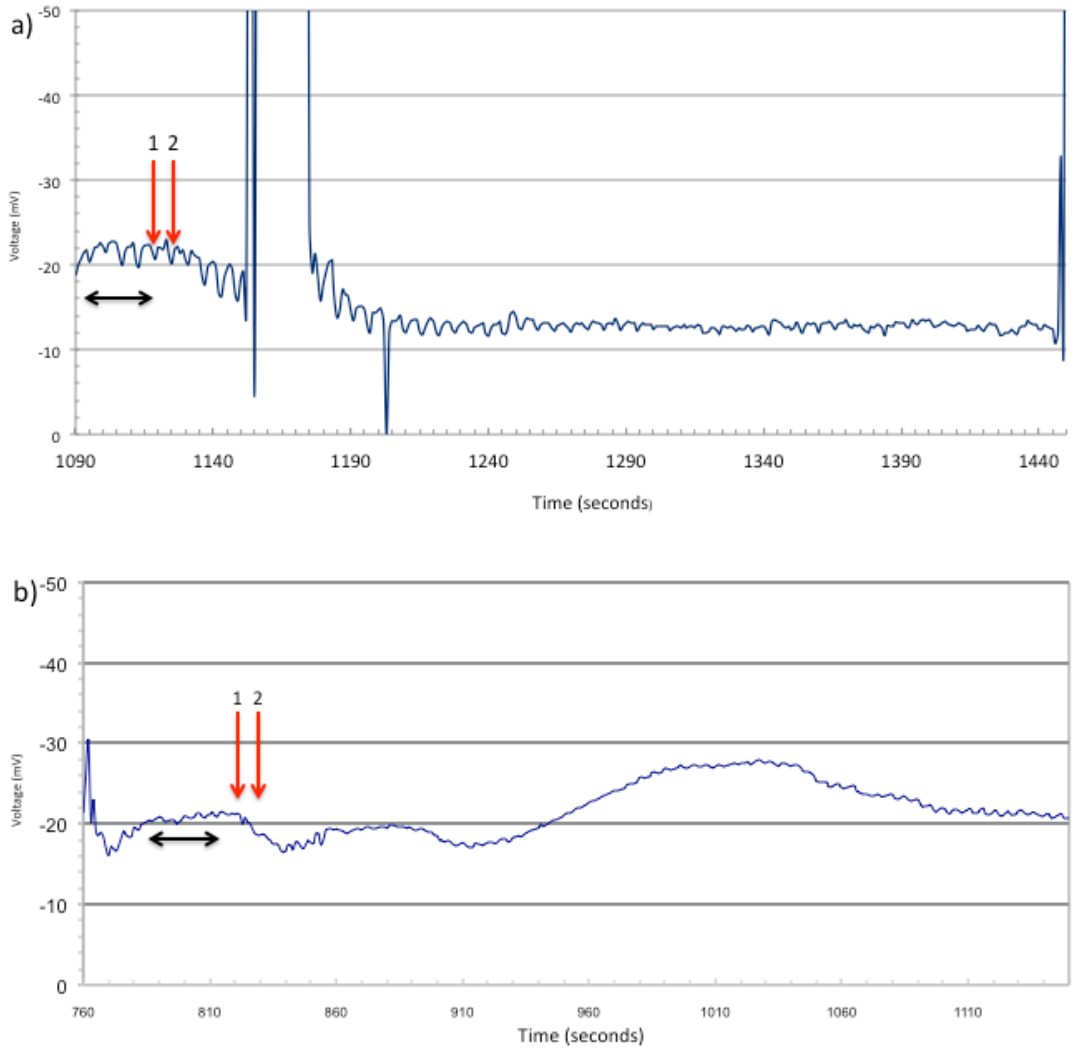


Figure 4.4-5: Examples of traces that were excluded where a) the trace was unsteady 50-80 seconds after perfusion of the ZCl/Iso solution and b) the trace deflected both upwards and downwards. The black arrow illustrates the basal measurement, the 1st red arrow where the Ringers solution was discontinued, and the 2nd red arrow when ZCl/Iso solution was commenced

If the trace fulfilled the acceptability criteria, the delta ZCl/Iso was calculated by subtracting the mean measurement after 300 seconds of perfusion of ZCl/Iso from the mean of the stable part of the trace between commencing ZCl/Iso perfusion and 50 seconds.

When calculating the change in values (basal values and measure of chloride secretion) between pre and post dosing visits:

- mean difference = mean post dose value - mean pre-dose value
- most negative = most negative post dose value - most negative pre dose value.

4.5 Statistics

The bronchoscopy subgroup was included in the main trial as observational and opportunistic studies that were underpowered to show a statistical difference.

The analysis of the trial data of special interest for this thesis was performed by myself, using Prism Version 6 software (GraphPad). The outcome between active and placebo groups was assessed with Mann-Whitney U test (normality not assumed due to small data size). Correlation between outcome measures was evaluated using non-parametric spearman rank correlation. The null hypothesis was rejected at $p<0.05$

4.6 Results

4.6.1 Bronchoscopy subgroup demographics

Figure 4.6-1 illustrates the profile of subjects recruited into the bronchoscopy subgroup. The bronchoscopies for 4 patients; 2 active and 2 placebo, were performed outside the timeframe, due to ill health and subject availability. The values for the 2 subjects in the active arm were omitted from the final analysis, and the patients on placebo arm were included on the basis that 0.9% saline would be unlikely to influence bronchial electrophysiology. There were paired pre and post dosing bronchoscopy LAPD measurements in 17 patients (active 10, placebo 7).

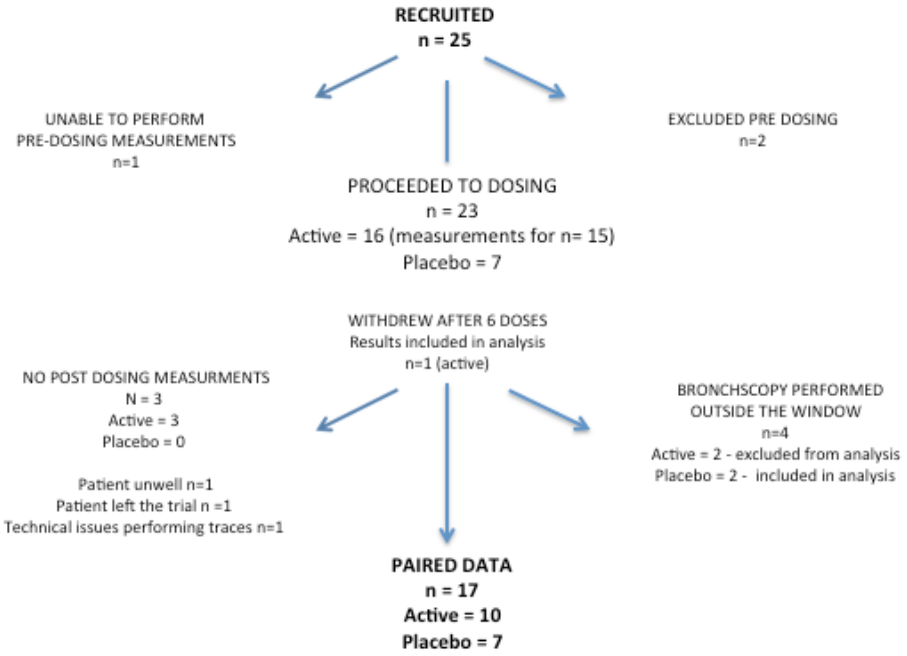


Figure 4.6-1: Summary of the patients in the bronchoscopy subgroup

The patient demographics are summarised in Table 4.6-1.

	Placebo		Active	
	Bronchoscopy Subgroup	**MDT**	**Bronchoscopy Subgroup**	**MDT**
Number (n)	7	54	16	62
Age (years) mean (SD)	28.9 (13.4)	26.0 (13.0)	21.5 (10.5)	23.6 (10.8)
Sex				
Female n (%)	4 (57)	25 (46)	10 (62.5)	31 (50)
Male n (%)	3 (33)	29 (54)	6 (37.5)	31 (50)
FEV_1 (% predicted) mean (SD)	67.0 (10.5)	69.0 (9.0)	71.4 (8.8)	69.9 (11.1)
Mutation class				
F508del/F508del	3 (42.8)	26 (48)	10 (62.5)	31 (50)
F508del/Other	4 (57.2)	28 (41)	6 (37.5)	31 (50)

Table 4.6-1: Summary of patient demographics in the bronchoscopy group and the entire MDT. *The age/ sex/ FEV_1% predicted and genetic mutation of patients in the placebo and active group are documented. Data are mean (SD) or n (%) unless otherwise stated.*

4.6.2 Adverse events related to bronchoscopy visits

There was one serious adverse event considered by the DSMB likely related to a bronchoscopy visit which was the patient being admitted to their local hospital with a respiratory exacerbation within 24 hours of the procedure.

4.6.3 Results of primary outcome (FEV_1 % predicted) measure in patients in the bronchoscopy subgroup

As this was a small subgroup it was not expected that significant treatment effects would be observed, however a treatment effect was seen for the primary outcome measure with the active group demonstrating a significant improvement in FEV_1% compared to the placebo group; 14.2 % $p<0.01$. The relative changes demonstrated between the 2 groups were +3.35% in the active group compared to -11.16% in the placebo group illustrated in Table 4.6-1. These discrepant findings compared to the main trial group are discussed below.

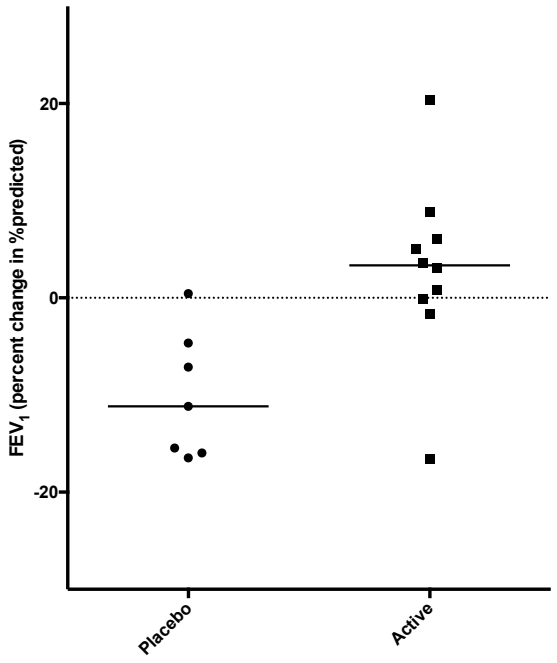


Figure 4.6-2: FEV_1% predicted response to active product and placebo in bronchoscopy subgroup, demonstrating a greater improvement in the group on active treatment ($p<0.01$). *The horizontal line illustrates the median*

4.6.4 LAPD measurements

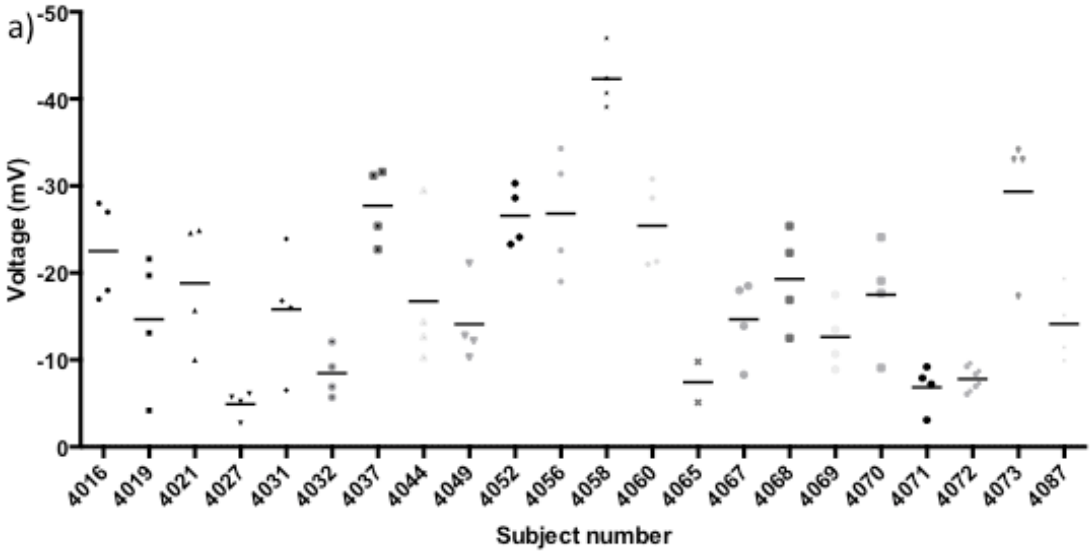
Total number of traces for analysis were 67 (both traces with basal measurements performed at the 4 locations at the carina (once per procedure), and traces performed distally including basal values and response to ZCl/Iso (performed 3x per procedure). The number of acceptable traces for analysis of the basal measurements in the carina and distal bronchi were 98.5% and for the analysis of chloride secretion was 64.7%.

4.6.4.1 Pre-dosing measurements

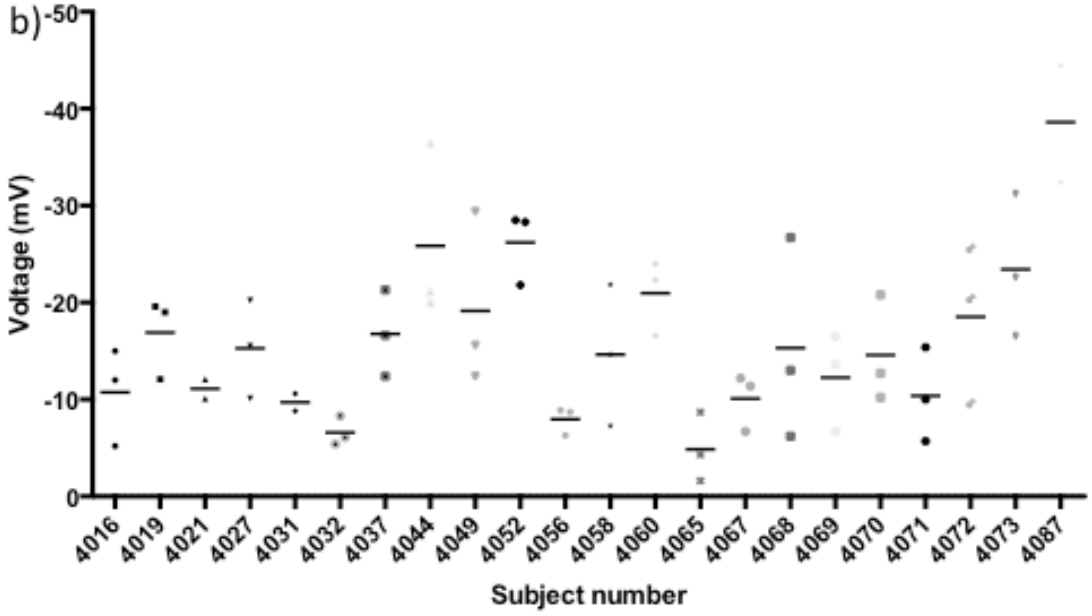
Basal carina and distal measurements: All pre-dosing measurements on subjects who progressed to dosing were included in this analysis (n=22).

Delta ZCl/Iso: Only the measurements taken from subjects in which there are paired pre and post treatment data were used in this analysis (n=17). The results for the measurements recorded pre-dosing; basal carina values, distal basal values and the chloride secretion measured by delta ZCl/Iso values, for each individual, are summarised graphically in Figure 4.6-3. Both the mean, and the most negative delta ZCl/Iso values are illustrated. Each patient is represented by the candidate number on the x-axis. The individual traces are displayed as data points, with the horizontal bar indicating the mean and the SD. In graph c and d, the scale is adjusted to account for both positive and negative changes in the PD.

Basal carina measurements



Basal distal measurements



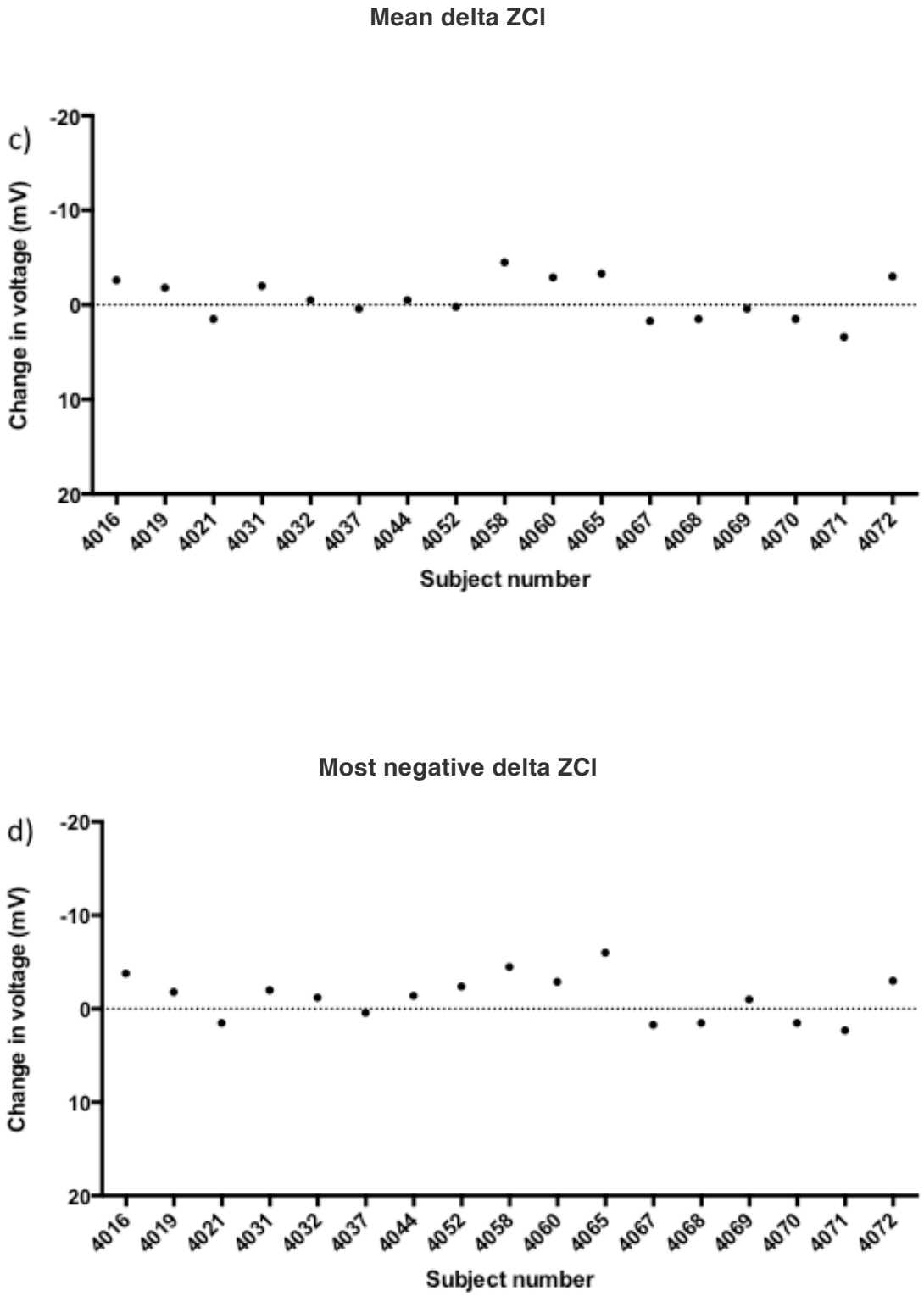


Figure 4.6-3: Pre dosing LAPD measurements. These graphs display the pre-dosing LAPD values for each patient obtained at a) the carina (4 measurements at the left lateral/ right lateral/ anterior and posterior locations) b) distal basal values (3 different locations at approximately 5th generation bronchi) and c) the mean change in voltage in response to ZCl/Iso and d) the most negative change in voltage in response to ZCl/Iso.

The mean and most negative basal values at the carina were compared with corresponding values taken in the distal airways, illustrated in Figure 4.6-4, showing no difference between the measurements and the anatomical site using; p=0.07 and p=0.1 (Wilcoxon matched-pairs signed rank test).

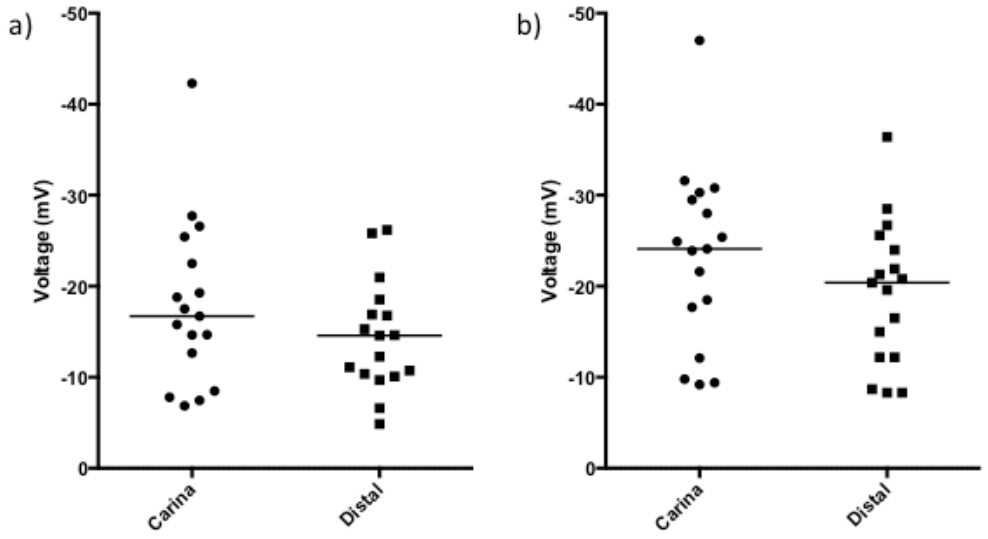


Figure 4.6-4: Comparison of basal values at the carina and the distal airway demonstrating no significant difference when comparing a) the mean values and b) the most negative values. *The horizontal line indicates the median.*

The measurements at the carina for individual patients were found not to correlate with the distal basal values either when comparing the mean values (r = 0.19 p=0.42) or the most negative values (r= 0.22 p=0.33) (Figure 4.6-5)

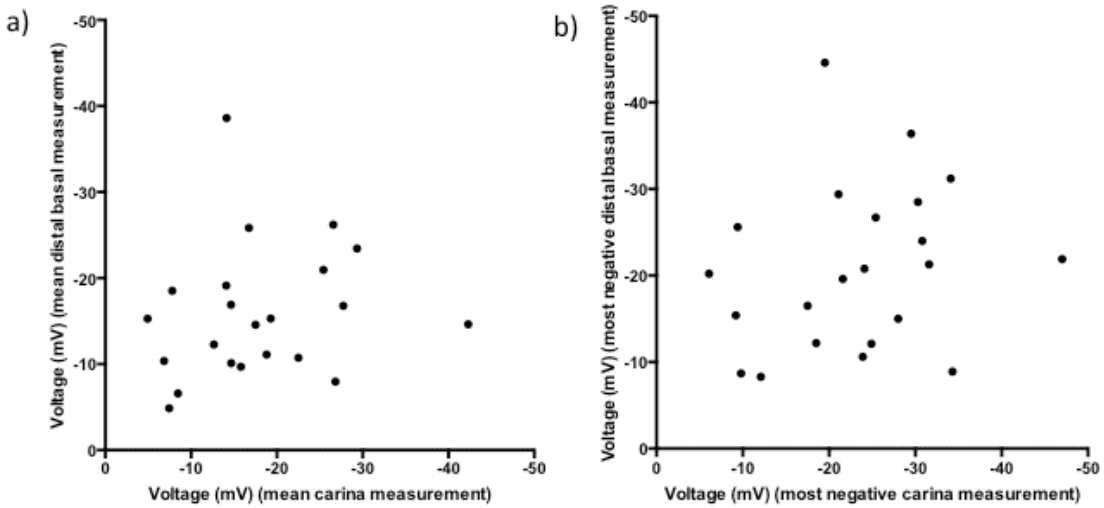


Figure 4.6-5: Comparison between measurements made at the carina and at the distal bronchi. a) comparison of the mean values at the carina with the mean distal value and b) the most negative values at the carina compared to the most negative distal values.

4.6.4.2 Comparison of LAPD measurements between active and placebo groups

LAPD measurements following treatment with active product and placebo were compared. Both the mean values and the most negative values for the different parameters were compared. No difference was seen between the change in voltage (mV) between the active (n=10) and placebo (n=7) groups at the carina (comparison of mean values p=0.40 and most negative values (p= 0.23) (Figure 4.6-6) or the distal basal measurements (comparison of mean values p=0.94 and most negative values p> 0.9) (Figure 4.6-7). The basal LAPD measurements are thought to largely reflect sodium transport via ENaC, with a lower (less negative value) seen in non-CF. A change in these values has not previously been reported following gene therapy.

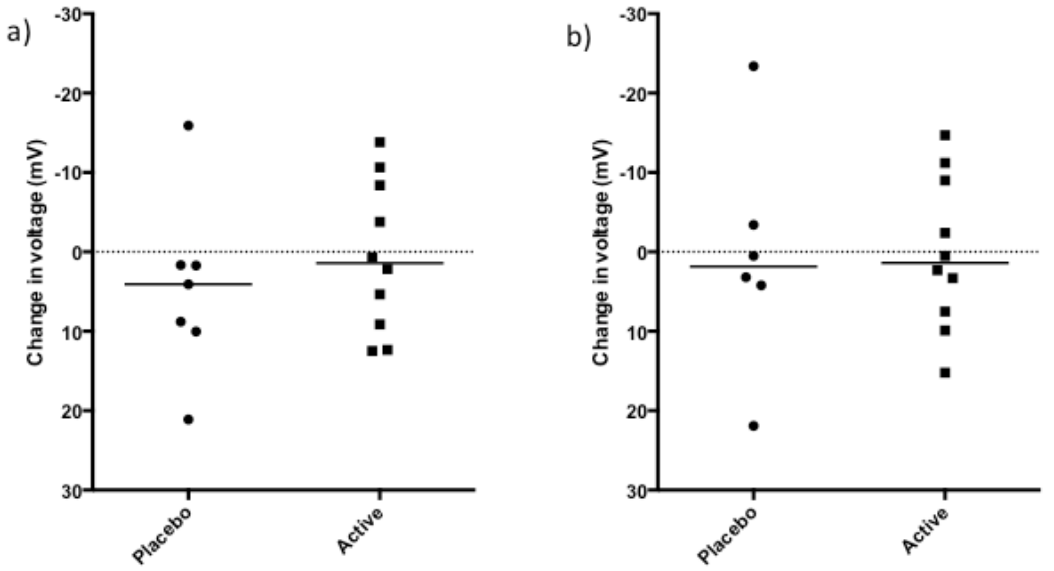


Figure 4.6-6: Comparison between the change in basal measurements in the carina between active and placebo groups post dosing. a) mean values and b) most negative values. A more positive change is considered to beneficial and towards non-CF values. *The horizontal line indicates the median*

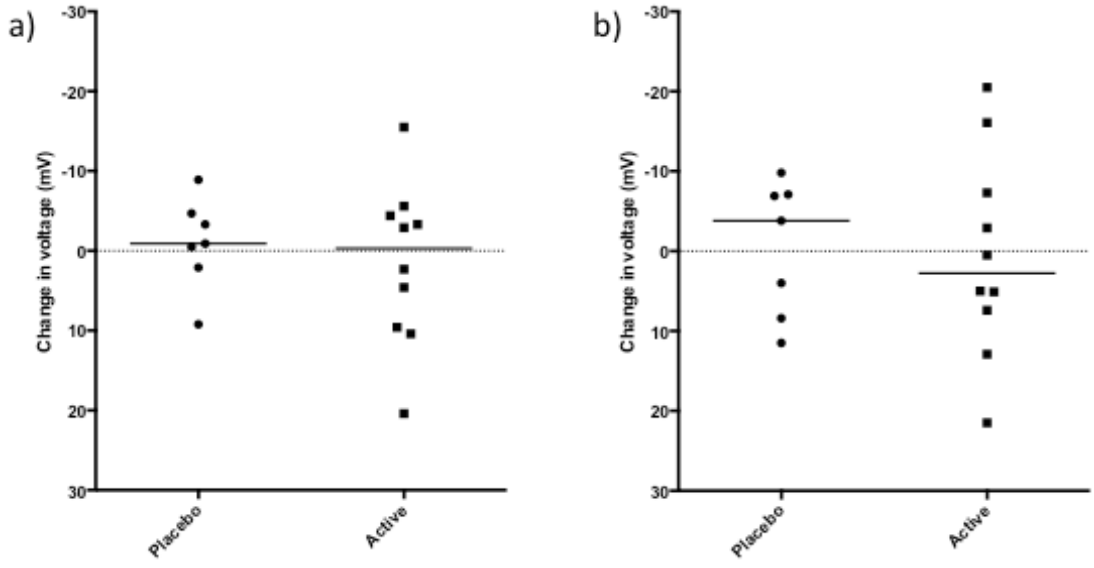


Figure 4.6-7: Comparison between the change in distal basal measurements between active and placebo groups post dosing. A more positive change is considered beneficial and towards non-CF values a) mean values and b) most negative values. *The horizontal line indicates the median*

The change in voltage following perfusion of ZCl/Iso (chloride secretion) was compared between those who had received active product versus those who had received placebo (Figure 4.6-8). A more negative value is associated with improved chloride secretion, consistent with improved CFTR function. A significant difference was demonstrated between the mean values of all interpretable traces from each patient (p=0.03) (Wilcoxon matched-pairs signed rank test). The active group had a median change of -1.3mV (range +4.0 to –5.8mV) compared to +3.1mV (range +9.3 to –1.2mV) in the placebo group. Comparison of the most negative values did not reach statistical significance (p=0.08), with a median delta ZCl/Iso change of – 2.8mV (range +4.0 to –16.8mV) in the active group compared to median +2.6 mV (range + 9.3 to – 1.2mV) in the placebo group. 6 out of 10 active-treatment patients had values more negative than the largest placebo response.

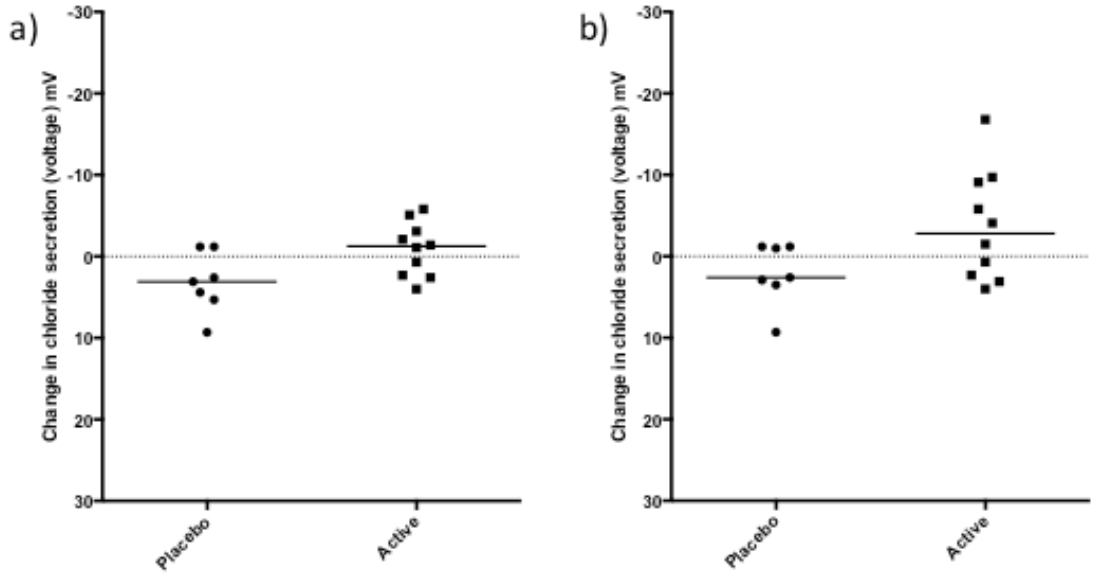


Figure 4.6-8: Comparison between basal delta ZCl/Iso at distal locations between active and placebo groups post dosing. A more negative change (indicating chloride secretion) is considered to beneficial and towards non-CF values. a) mean values (p=0.03) and b) most negative values (p=0.08). *The horizontal line indicates the median.*

4.6.5 Relationship between LAPD measurements and markers of disease severity and inflammation

4.6.5.1 Relationship at baseline

Physiological outcome measures

For all comparisons the mean measurement, and the most negative value from all traces have been used. The basal reading at the carina, the distal basal value and delta ZCl were compared to the FEV_1% predicted and LCI values performed on the screening visit. The results are displayed in Figure 4.6-2.

LAPD measurement		**Outcome**			
Position	**Value**	**FEV_1%**		**LCI**	
		r	p value	r	p value
Carina	Mean	0.22	0.33	-0.35	0.12
	Most negative	0.28	0.21	-0.32	0.16
Distal basal	Mean	0.35	0.1	0	0.98
	Most negative	0.43	0.04*	0.08	0.72
Delta ZCl	Mean	-0.08	0.77	-0.44	0.09
	Most negative	-0.1	0.7	-0.25	0.35

Table 4.6-2: Correlation between LAPD values and markers of disease severity measured by FEV_1% and LCI performed on the screening visit. *Both the r (Pearson correlation coefficient) and p value are tabulated, with those that are statistically significant indicated with a **

A significant correlation was seen between the most negative basal measurements obtained in the distal airway when compared to FEV_1%, suggesting that those with more severe disease (lower FEV_1) had a higher (more negative) basal measurement, reflective of underlying ENaC over-activity secondary to loss of CFTR modulation of this ion channel. Whilst it didn't reach statistical significance, there was a close association between the mean basal distal measurement and FEV_1% predicted Figure 4.6-9.

No correlations were detected between any of the other parameters derived from LAPD measurements and FEV_1 or LCI.

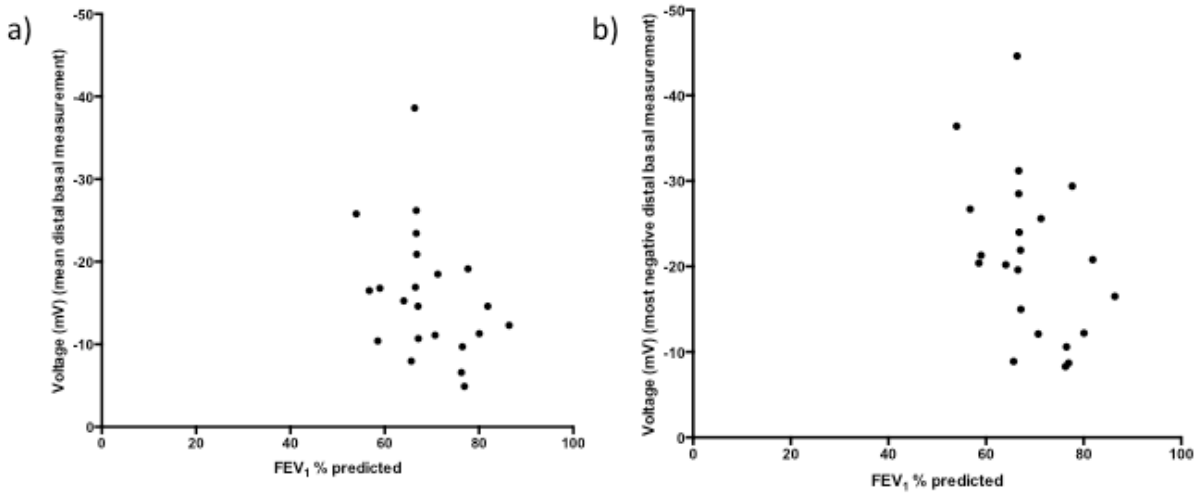


Figure 4.6-9: Correlation between patient's baseline FEV_1% predicted and a) mean distal basal value, revealing a trend in correlation, which was not significant (p=0.1) and b) the most negative distal basal value which was significant (p=0.04)

Measures of inflammation in serum and sputum

At baseline, there was no correlation between any parameters derived from LAPD measurements and any measure of inflammation in the serum and sputum

Table 4.6-3). As none of the correlations were significant, correction for multiple comparisons was not applied.

a)

LAPD measurement		Serum									
Position	Value	CRP		WCC		ESR		Calprotectin		IL6	
		r	p	r	p	r	p	r	p	r	p
Carina	Mean	0.12	0.66	0.15	0.59	-0.04	0.86	-0.01	0.97	-0.21	0.38
	Most negative	-0.04	0.9	0.12	0.67	-0.16	0.5	-0.13	0.58	-0.3	0.2
Distal basal	Mean	0	1	-0.43	0.11	0.11	0.63	-0.03	0.9	0.03	0.89
	Most negative	-0.02	0.95	-0.4	0.14	0.12	0.6	0	1	0.02	0.94
Delta ZCl	Mean	-0.18	0.5	0.67	0.11	-0.11	0.63	0.18	0.45	-0.16	0.49
	Most negative	-0.02	0.93	0.44	0.1	0.03	0.91	0.24	0.3	-0.03	0.9

b)

LAPD measurement		Sputum							
Position	**Value**	**TCC**		**Neutrophils**		**Calprotectin**		**IL8**	
		r	p	r	p	r	p	r	p
Carina	Mean	-0.14	0.67	-0.38	0.22	-0.02	0.94	-0.3	0.4
	Most negative	-0.09	0.79	-0.29	0.35	-0.03	0.92	-0.31	0.38
Distal basal	Mean	0.18	0.59	0.18	0.57	-0.48	0.11	-0.52	-0.39
	Most negative	0.19	0.57	0.06	0.85	-0.48	0.11	0.12	0.26
Delta ZCl	Mean	0.06	0.86	0	1	0.01	0.98	0.32	0.37
	Most negative	0.21	0.53	0	0.99	-0.07	0.82	0.33	0.36

Table 4.6-3: Correlation between LAPD values and measures of inflammation in a) serum b) sputum. *Both the r (Pearson correlation coefficient) and p value are tabulated, with those that are statistically significant indicated with a *.*

4.6.5.2 Relationship between change in LAPD measures and measures of airway disease following gene therapy

As there was only a significant treatment effect in mean chloride secretion (delta ZCl/Iso), only these values were compared with changes in outcome measures reflecting disease status and inflammation.

Physiological outcome measures

An increase in FEV_1% and decrease in LCI is consistent with an improvement in disease. No correlation was demonstrated between the change in chloride secretion and either change in FEV_1% predicted (r=0.24, p=0.52) or change LCI (r=0.04, p=0.92) between pre and post dosing values in the active group (Figure 4.6-10)

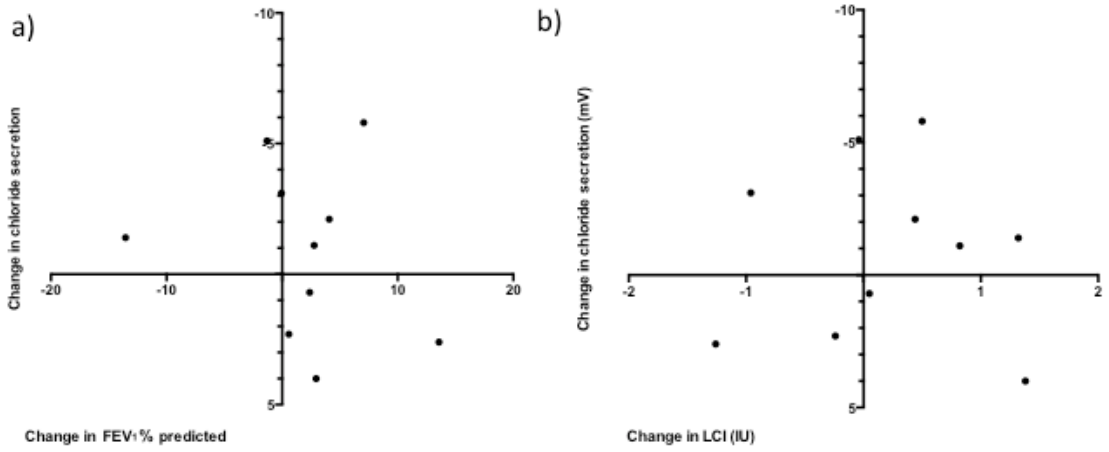


Figure 4.6-10: Relationship between change in chloride secretion pre and post dosing (with a more negative value considered to be beneficial) in the active group and a) FEV_1% predicted (increase is beneficial) and b) LCI (decrease is beneficial) showing no correlation between either parameter.

Measures of inflammation in serum and sputum

As discussed (Chapter 3), there was no significant change levels of inflammation in serum and sputum samples pre- and post- gene therapy, and no correlation was shown between the change in these parameters and the change in chloride secretion Table 4.6-4.

LAPD measurement		Serum									
Position	Value	CRP		White Cell Count		ESR		Calprotectin		IL6	
		r	p	r	p	r	p	r	p	r	p
Delta ZCl	Mean	0.07	0.9	0.07	0.9	0.1	0.8	0.03	0.95	0.08	0.84
	Most negative	0.11	0.84	0.1	0.81	0.1	0.81	0.22	0.58	0.27	0.49

LAPD measurement		Sputum									
Position	Value	Sputum weight		Total Cell Count		Neutrophils		Calprotectin		IL-8	
		r	p	r	p	r	p	r	p	r	p
Delta ZCl	Mean	-0.4	0.75	0.6	0.35	0.21	0.77	-0.2	0.78	0.2	0.92
	Most negative	-0.4	0.75	0.5	0.45	0.36	0.63	-0.1	0.95	0.2	0.92

Table 4.6-4: Relationship between change in chloride secretion pre and post dosing in the active group and secondary outcome measures revealing no significant correlations: *Both the r and the p value for each comparison are documented.*

Relationship between change in LAPD measurements and change in CFTR cDNA

I analysed only those patients receiving active treatment. There was no statistically significant correlation between vector- specific DNA measured from the bronchial epithelium with the total chloride secretion (delta ZCl/Iso) measured in response to active treatment (r=-0.02, p=0.98) (Figure 4.6-11)

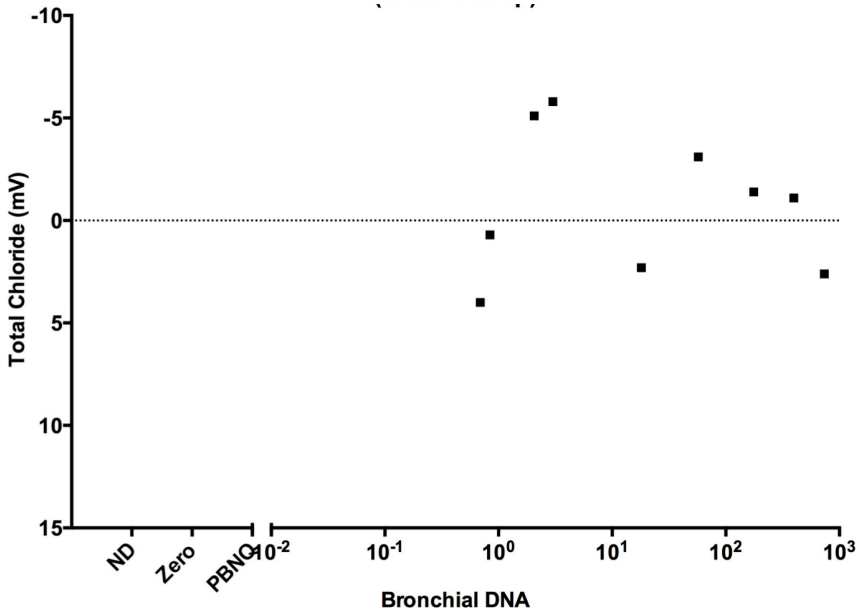


Figure 4.6-11: Graph plotting the correlation between bronchial cDNA and LAPD choride response following active treatment

4.6.6 Analysis of variability of LAPD measurements

4.6.6.1 Intra-subject variability

Within measurements coefficients of variation (CV)%: For all subjects, the mean intra-subject CV% for baseline measurements taken pre-dosing at the carina and distal airway respectively were 31.37% and 35.7%.

Between measurement CV%: For the 7 patients who had received the placebo, I calculated the between measurement intra-subject CV% by comparing the values obtained pre-dosing with those obtained post dosing (approximately 12 months apart). The CV% between mean values at the carina was 31.7%, and between most negative values 25.2%. The CV between mean distal basal values was 21.5% and between the most negative values was 27.8%.

The intra-subject variability was thus comparable for both between measurements performed at the same time point and within measurements performed on different occasions.

4.6.6.2 Variability and disease severity

The airway of CF patients with severe disease is characterised by patchy inflammation and mucus deposition. It was thought that more severe disease might affect the variability of the LAPD measurements, with mucus disrupting the contact between the catheter and the epithelial surface, and localised inflammation affecting epithelial cell integrity heterogeneously throughout the airway. To assess this, I determined whether the CV of LAPD measurements was related to FEV_1 and LCI as measures of disease severity.

Within measurements: Neither the CV% of the measurements taken at the carina or the distal basal measurements were found to correlate with the FEV_1% predicted or LCI value (**Error! Reference source not found.**)

CV (%) site	**Disease severity**			
	FEV_1(% predicted)		**LCI**	
	r	p	r	p
Basal carina	0.03	0.91	-0.01	0.97
Distal basal	-0.05	0.85	-0.24	0.38

Table 4.6-5 Correlation between markers of disease severity measured by FEV_1% and LCI and the within measurement CV% of basal measurements performed at the carina and in the distal airway. *Both the r (Pearson correlation coefficient) and p value are tabulated*

Between measurements: The CV% of the mean an most negative measurements taken pre and post dosing at the carina and the distal basal measurements in the 7 patients in the placebo group were compared to markers of disease severity (FEV_1% and LCI) taken at the screening visit. There was no correlation between the CV% of the mean or most negative values at the carina or the distal basal measurements and FEV_1% and LCI

CV (%) site	**Disease severity**			
	FEV_1(% predicted)		**LCI**	
	r	p	r	p
Basal carina	-0.57	0.2	-0.26	0.66
Distal basal	-0.36	0.44	0.37	0.5

Table 4.6-6: Correlation between markers of disease severity measured by FEV_1% and LCI and the between measurement CV% of basal measurements performed at the carina and in the distal airway. *Both the r (Pearson correlation coefficient) and p value are tabulated,*

These results suggest that the variability of LAPD measurements is not dependent on the disease status of the patient.

Using this variability data; power calculations (GraphPad StatMate 2) revealed that in order to determine a difference basal values of 10mV a sample size of 16 patients in each group (32 in total) (95%CI) would be required. For a difference in chloride secretion of 5mV (95% CI), a sample size of 14 patients in each group (28 in total) would be required.

4.6.7 Relationship between NPD and LAPD measurements

The relationship between the NPD and LAPD measurements of subjects (n= 5) who participated in both subgroups was reviewed by Dr Waller in his PhD thesis. There was no relationship between the basal values. A significant correlation was identified at baseline between the nasal and bronchial chloride response. Although the numbers were small, this suggests that patients with residual chloride secretion have so in both airway sites. Following gene therapy only 5 patients had matching NPD and LAPD measurements. A trend was suggested between improved chloride in both nasal and bronchial epithelium however the numbers were too small to draw firm conclusions [196].

4.7 Discussion

This chapter focused on the LAPD results obtained from the subjects in the bronchoscopy subgroup of the Mutlidose Trial. To our knowledge there are no studies that have compared CFTR ion transport function in the lower airways with markers of disease severity and inflammation, or assessed its use as a functional endpoint assay for novel therapies in CF aiming to correct the basic defect. Analysis of the pre-dosing LAPD measurements demonstrated a correlation between a higher (more negative) distal basal LAPD measurement and a lower FEV_1 % predicted, but not with other outcome measures. Treatment with gene therapy resulted in a small but statistically significant improvement in lower airway chloride secretion, however this did not correlate with improvement in FEV_1 % predicted or other markers of disease severity or inflammation. Finally, it was shown that the variability of LAPD measurements was high, but not dependent on disease

severity. These results are discussed in more detail below, but must be interpreted with caution because the numbers are small and the magnitude of the changes in chloride transport are not large.

4.7.1 Pre dosing LAPD measurements

These results replicated previous studies, showing near zero (mean -0.6mV) chloride secretion in CF subjects; indicating that the technique was reliable and that subjects were representative of the disease group [195]. However, in contrast to the published literature, the basal measurements at the carina were not higher (more negative) than those taken distally, which may be due to the small sample size. As expected, no correlation was found between carina and distal measurements on an individual basis, likely reflecting the variability in expression CFTR throughout the lung. In the proximal airway structures CFTR chloride channel is thought to be expressed at low levels [289]. In the distal airway, the highest levels of CFTR expression have been demonstrated in the respiratory bronchioles as compared with proximal bronchioles (two fold) and alveoli (five fold) [290].

4.7.2 LAPD response to gene therapy

No treatment effect was observed in the change in basal values at the carina and the distal airways. This was expected because, as previously discussed, correction of the basal values (reflecting sodium hyperabsorption) is thought to require CFTR correction in almost all epithelial cells, which was not anticipated from delivery of lipid-mediated gene therapy. The small but statistically significant treatment effect change in chloride secretion of -4.4mV (more negative value towards non-CF values, indicated successful gene transfer. These results were not accompanied by increased vector specific mRNA in the active group, as the levels were below the limits of detection, (Chapter 3) however, previous studies have suggested that PD measurements are more sensitive than measurement of vector mRNA to detect evidence of gene transfer [284].

The difference between the change in chloride secretion between both groups was largely as a result of the placebo group becoming more positive (+3.1mV) compared to the active group who only had a modest increase in chloride secretion of -1.2mV. This was a small patient group (active = 10, placebo = 7) and it is possible that in this size group, variations in only a few measurements may alter the interpretation. Looking at the individual responses, 6 out of 10 active-treatment patients had values more negative (towards non-CF values) than the largest placebo response.

There are inherent limitations in LAPD measurements, which affect the interpretation of these results. It is a technically complex procedure, resulting in a high variability in the measurements,

with a within CV in the distal airway of 37% (discussed below). During the procedure, only a small area of the bronchial epithelium is being assessed. In subjects with CF, the presence of mucus and airway secretions prevents even distribution of the drug throughout the airway, and PD measurements may record that had a lesser degree of transfection, thus underestimating the response.

It is also important to consider that the improvement seen in FEV_1 % in the active group (reported in Chapter 3) may not be a direct result of gene therapy and successful gene transfer, but a non-specific reaction to the plasmid complex itself, especially in the light of the mRNA results and the lack of electrophysiological changes detected in the nasal subgroup. This would be unexpected, as liposome alone has shown no evidence for producing physiological improvements in the airways of non-CF or CF subjects, and plasmid DNA is more commonly associated with deleterious effects. Furthermore, there was no evidence of lipid accumulation in the airways of subjects on active treatment. Due to ethical reasons it was not possible to administer a CFTR plasmid/liposome complex as the placebo due to its unknown effect on the lung when multiple doses are delivered and 0.9% saline was used instead, therefore a direct comparison is not possible.

In order to evaluate whether the change in chloride secretion reported in the lower airway was a true treatment effect of lipid-mediated gene therapy, further studies in larger groups of patients are required. Using larger or more frequent doses and/or performing LAPD measurements in more sites would increase detection of CFTR restoration. Ultimately, it may be that the lipid- based vector does not result in adequate levels of gene expression to be detected by LAPD measurements

4.7.3 Relationship between CFTR function and disease severity

Interestingly, this study revealed that at baseline, the most negative distal basal measurement correlated with more severe disease measured by FEV_1 %. No correlation was seen between the distal basal measurements and LCI or inflammatory mediators and there was no relationship between chloride secretion in the distal airway or measurements at the carina and disease severity or inflammation.

As the group size was small, it is difficult to draw firm conclusions from these results, however the correlation between the most negative distal basal measurements and FEV_1 is a new finding. This supports studies that showed a correlation between more negative basal NPD values and larger

amiloride response with worse respiratory disease [245]. The basal hyperpolarisation (more negative values) of the bronchial mucosa reflects disinhibition of ENaC (caused by absent CFTR function), causing increased sodium absorption. The correlation between distal basal LAPD and FEV_1 was not seen with LCI. As FEV_1 is thought to represent airway damage in the proximal airways, compared to LCI, which detects disease in the distal airways (much more distal than the LAPD measurements), the correlations that we saw would be anticipated. However, confirmation of the relationship between FEV_1 and basal distal measurements is required in a larger group, particularly since this was not seen in proximal measurements

The Multidose Trial demonstrated a significant improvement in FEV_1 in both the trial group as a whole, and in the smaller bronchosopy subgroup, accompanied by a trend towards improvement in other outcome measures of disease. The change in FEV_1 was an unexpectedly larger difference than that demonstrated in the trial as a whole; and as the baseline demographics of the subgroup were comparable to that of the overall trial, it is most likely to be a chance finding due to the small numbers in the group. As chloride secretion was the only electrophysiological measurement to demonstrate a treatment effect, changes in this parameter were compared to changes in outcome markers of disease and inflammation revealing no significant correlations.

The lack of correlation between chloride transport and disease severity both at baseline and following CFTR restoration may be due to several, not mutually incompatible, reasons, either due to the limitations of the outcome measures, the patients or potentially because chloride ion channel transport does not directly correlate with airway disease:

- LAPD measurements may be too insensitive to perform correlation studies. The variability in measurements was high, and may not allow subtle comparisons to be made with a feasible number of patients (estimated 32 or 28 subjects required in total to reveal a significant difference in basal and chloride measurements respectively).

- The inclusion criteria for the trial required FEV_1% to lie between 50 and 90% thus automatically excluding those at the edge of the spectrum of disease severity in whom a correlation may be most evident.

- There are also limitations in interpreting the outcome markers of disease severity. Many of these measures are insensitive, non-specific, and are time dependent measures which may influence the results. FEV_1 measurements also have some variability from test to test, with short term variability reported in the literature as (SD) 6.3%, and dependent upon many variables including time of day, mood and tiredness [291]. The variability of inflammatory

biomarkers in sputum is reported to be high, and how these markers relate to disease severity and longitudinal changes in lung function is still being explored [292].

- It is known that a variety of other factors influence both CFTR function and disease severity e.g. age, genotype, infectious status, pancreatic sufficiency status, modifier genes, environmental influences, tobacco and air pollutants.

- As considered above, the treatment effect in FEV_1 may not have been a direct response to gene therapy, but instead to the plasmid complex, although unlikely this cannot be excluded.

- It may be that chloride secretion is not the principal force in determining the severity of airway disease. The correlation between basal distal values and FEV_1 implicate ENaC hyperactivity in disease pathogenesis, likely by increased sodium absorption causing mucus dehydration and increased viscosity. The extent to which ENaC contributes to disease is an ongoing debate [293, 294]. Furthermore, CFTR dysfunction in immune cells (Section 1.2.3.3) may play a larger role in disease progression than expected, and/or a dysregulated inflammatory response as explored in the next chapter. A combination of these influences, rather than chloride secretion alone may lead to airway damage. Supporting this theory are results from studies exploring the efficacy of Ivacaftor, which produced large changes in FEV_1 but only minimal (approximately 35%) improvement in chloride response.

Despite being the largest data set of traces to date, the numbers are still small, and to truly assess for a correlation between CFTR function measured by LAPD measurements and disease severity, more directed and larger trials are required although practically this will be hard to achieve.

4.7.3.1 Variability of LAPD measurements

The pre-dosing within-subject variability of LAPD measurements (basal carina, basal distal and delta ZCl/Iso) was shown to be higher than that demonstrated in NPD measurements, (between 9 and 15% for NPDs performed on different days) likely reflecting the complexity of the procedure and the increased presence of mucus and inflammation on the surface of the bronchial epithelium [295, 296]. It was thought that the variability of measurements may increase in subjects with more severe disease. Inflammation in the nasal epithelium is known to cause a ‘blunted’ PD response, characterised by a lower basal value and a flatter responses to the perfusion Ringers Amiloride

and ZCl solutions [192]. This was not demonstrated, with no correlation seen between CV% and disease severity as measured by patient's baseline FEV_1 % predicted and LCI measurements, although it must be stressed that the numbers are small. These results were replicated in the group of patients who had been randomised to receiving the placebo with a similar variability calculated between the measurements obtained pre dosing with those performed approximately 12 months later, where again no correlation was seen between the variability of measurements and disease severity. This may be because in comparison to NPD measurement, in LAPD, the catheter is placed under direct vision, thereby improving the chance of better contact between the device and the epithelium.

Despite this, there is still a large inherent variability in the measurements. In this study, both the mean values, and the most negative PD values were recorded for each parameter, as it is unknown which provides the most useful readout measurement. Averaging the PD measurements reduces the variability between measurements performed at different sites, however it has been shown that this may underestimate CFTR function [297, 298]. The most negative PD measurement is thought to reflect sodium transport more than CFTR, therefore may miss a chloride response [299]. In this study, both measurements have revealed different information, suggesting that both should continue to be analysed. LAPD measurements can used to successfully discriminate between non-CF and CF subjects, however it does not appear to be sensitive to small changes in CFTR expression *in vivo*, and alternative assays are required [195].

4.7.4 Limitations

There are limitations to this study, most of which have been mentioned. The practical difficulties involved in performing LAPD measurements, and the necessarily invasive technique means that the numbers in this study are small.

4.8 Summary and future work

Ultimately, this study showed that there was an improvement in both FEV_1 and LAPD chloride secretion (thought to indicate successful transfer of CFTR to the airway epithelium) following multiple doses of gene therapy, however there was not enough evidence to support the hypothesis that increased expression of CFTR, as measured by LAPD measurements, has a direct, inverse association with markers of airway disease, and that changes following repeat dosing of CFTR

gene therapy correlated with an improvement in clinical markers of airway disease. Pre-dosing, distal basal measurements were shown to correlate with disease severity as measured by FEV_1% predicted, but not LCI, as would be expected given the location of the measurements, but no correlation was seen with other values. The small but significant change in chloride secretion did not correlate with changes in outcome markers of designed to measure inflammation or disease severity.

In order to fully establish whether a relationship exists between LAPD measurements and CFTR function, it would be useful to perform more measurements to see if the results are replicated in a larger group, or with an intervention known to produce a big increase in ion transport. It would be particularly useful to treatment with ivacaftor, which has been demonstrated to restore CFTR function in the skin (sweat chloride concentrations halved), the nasal epithelium, and to produce a significant clinical benefit.

Since this study failed to confirm an effect of dysregulated chloride and sodium transport on CF airway disease, in the next chapter I have gone on to explore an alternative pathophysiological mechanism of disease, related to dysregulation of the immune response, as a prelude to the final results chapter exploring the other limb of the hypothesized bidirectional relationship between CFTR and inflammation, namely the effects of inflammation on CFTR.

5 CFTR function and the inflammatory response to bacterial challenge of nasal epithelial cells cultured at an air-liquid interface

5.1 Introduction

CF lung disease is characterised by recurrent bacterial infection and chronic airway inflammation resulting in an uncontrollable vicious cycle, ultimately causing severe airway damage. Previous results in this thesis have suggested that dysregulated chloride and sodium transport are not directly related to CF airway disease. As a consequence, I have explored an alternative mechanism in this chapter. There is growing evidence to suggest that the CFTR defect itself causes a dysregulated immune response, which contributes to disease progression, however to date the results from studies exploring this field are inconclusive. My predecessor at the Department of Gene Therapy, Dr Ives, undertook a study to further investigate the effect of CFTR dysfunction on the immune response by comparing the inflammatory response to bacterial challenge of CF cells with cells from subjects with non-CF CSLD (PCD) and healthy controls. This work provides pilot data for the study described in this chapter.

This section first provides a detailed review of the studies exploring the role of CFTR in immune regulation and an overview of the proposed mechanisms. This is followed by a description of the work preliminary work leading into the rationale and aims of this study.

5.1.1 CFTR and inflammation

It has been suggested that CFTR dysfunction alters the inflammatory response in one or more of several possible ways including; by stimulating an inflammatory response in the absence of infection, by stimulating an inflammatory response disproportionate to the level of the pathogen and failure actively to resolve the inflammatory response [300]. Infection and inflammation are hard to dissociate. This section provides an overview of the studies which have sought to establish the relationship between CFTR, infection and inflammation in human studies, cell culture systems and animal models followed by a review of the proposed mechanisms.

5.1.1.1 Clinical studies

Children with CF have been shown to have higher levels of pro-inflammatory cytokines, and

neutrophils in BAL than controls, even in the absence of apparent infection [301]. The ratio of neutrophils and IL-8 in BAL, in the presence of bacterial infection was significantly higher in subjects with CF than controls, disproportionate to the bacterial load [302, 303]. In addition, there is *in vivo* evidence of reduced quantities of anti-inflammatory molecules such as IL-10 and lipoxins in the CF airway compared to controls [304, 305]. However, the evidence is conflicting as other studies have demonstrated that BAL obtained from CF infants in the absence of infection had an inflammatory profile similar to those of control subjects, suggesting no inflammation without infection [306] [307] and in another study that some CF infants who have never had lung infection have no detectable inflammatory response [112].

There are several possible confounding factors. Firstly, inflammation and infective microbes are not evenly distributed throughout the lung. The levels detected in a BAL samples from a single region cannot be generalised to other lung segments [308], and infections may be missed [309]. BAL analyses were only performed at a single time point. An infection occurring before the study and successfully cleared could have accounted for the increase in inflammatory mediators. In addition, whilst microbial culture has been considered to be the 'gold standard' for the detection of microbes, it is estimated that only 1% of bacteria can be cultured [310]. As more sensitive detection techniques have been developed, such as 16S rRNA PCR pyrosequencing there is growing awareness that previously unidentified bacteria and microorganisms are present, which could be contributing to the inflammatory profile [311].

5.1.1.2 Cell culture

In vitro studies comparing healthy and CF cell cultures provide a useful model to explore the inflammatory profile of cells lines as both the quantity and exposure time to infection can be controlled, however, interactions between epithelial and immune cells are difficult to study, a weakness of these studies.

Basal levels of inflammatory cytokine release:

In primary respiratory epithelial cells, it was shown that IL-8 is produced in higher quantities in CF than in wild-type cells at basal conditions [103, 312, 313] [314], with an associated increase in activity of the transcription factor, NF-kB. Further studies have confirmed this, with evidence of increased NF kB activation, IL-8 production and other inflammatory mediators in CF airway epithelial cells at baseline compared to control [315]. However, other studies have showed no difference in the levels of cytokines between CF and non-CF cells when un-stimulated and even

reduced levels of IL-8 at baseline in CF airway epithelia leaving the results currently inconclusive [316].

Bacterial stimulation:

In vitro models exploring the inflammatory profile of CF cells following bacterial exposure have also produced variable results with some studies demonstrating a difference between CF and non-CF cells, others revealing a difference only under specific conditions whilst other studies have demonstrated none at all. These are summarized Table 5.1-1

Reference	Method	Result
Difference between CF and controls		
J Clin Invest 1995;96:2204–2210	IL- 8 measured in immortalized airway epithelial cells and CF epithelial cells following exposure to *PA* gene products.	*PA* gene products evoked a 4 fold higher IL-8 response in the CF cell line compared to its corrected cell line
J Allergy Clin Immunol 1999;104:72–78	Immunofluorescence and ELISA for IL-8 and 10 were used to compare the cytokine profile of CF and non-CF bronchial epithelial cells in culture.	NonCF cells secreted IL-10, with no IL-8 or IL-6. CF cells did not secrete IL-10 but produced more IL-8 and IL-6. Iimmunofluroescence data correlated
Am J Physiol Lung Cell Mol Physiol 2001;280:L493–502	Cytokine response to *PA* measured in 2 pairs of human epithelial cell lines; CF and non-CF.	CF cell line more IL-8, IL-6 and GMCSF than non-CF, with the difference between the 2 increasing over time
Becker et al cytokine secretion by CF airway epithelial cells 2004	Non-CF and CF bronchial epithelial cells were cultured at an ALI. The cytokines measured at baseline, and when activated with TNFα, a synthetic TLR-2 agonist, *SA* and *PA*.	Neither groups expressed IL-10 or RANTES. CF cells produced more IL-8 post TNFα and synthetic TLR-2 and *SA* exposure. No difference following *PA* at baseline, but more in CF cells in the presence of human serum.
Am J Physiol Lung Cell Mol physiol 288:L471-L479,2005	*In vitro* activation of NF-kappaB and IL-8 levels were measured in CF and non-CF cells in response to *PA*	Elevated levels of NF-kB activation in CF cells, not in non-CF, at baseline and a transient increase following *PA* stimulation. IL-8 mRNA only higher in CF cells later.
No difference between CF and controls		
Am J Respir Cell Mol Biol 1998;19:210–215	IL-8 levels measured in non-CF, CF and CF cells corrected with CFTR at baseline and stimulated with TNFα or RSV infection	IL-8 response in both. No difference between the groups at baseline or following stimulation.
Am J Physiol 1999;276:C700–C710	Chemokines measured in CF and non-CF airway epithelia at baseline and in response to TNFα and IFN-λ	No difference in IL-8 or MCP-1. CF cells expressed less RANTES than non-CF cells which was restored when cells were corrected with CFTR.
Am J Respir Cell Mol Biol 1999;20:1073–1080	Cytokines and specific mRNA were compared in CF and non-CF cell culture at baseline and followingIL-1B stimulation.	CF cells that were engineered to express the WT-CF gene secreted more IL-8 than the CF cell.

Table 5.1-1A review of the *in vitro* studies designed to review whether CF cells are hyperinflammatory following stimulation.

Limitations in cell modeling systems likely contributed to these inconsistent results. Acquiring the cells by mechanically detaching them from their environment likely produces an inflammatory response, which may interfere with further measurements of the inflammatory mediators in experimental conditions. As the cells have been removed from the internal environment, they are not exposed to circulating cells and mediators, which may affect their responses.

- Cell suspension models do not accurately reflect *in vivo* conditions as the basolateral surfaces are exposed to the stimulus, rather than just the apical surface as in the intact airway, and they have a short life span therefore cellular activity may deteriorate over the course of the experiment.

- Cell culture systems provide a more accurate model of *in vivo* conditions (described in detail below) however they also have limitations. Firstly, a range of cell modeling systems are used in the studies, which have been shown when in direct comparison to produce different and variable results [317]. Also, when using immortalized cells, clones of cells are derived from a mixed population of primary cells, therefore the isolated clone represents only a subpopulation of the primary culture that may or may not have the same characteristics as the principle cell-type of origin. The process of culturing and passaging cells can also alter the expression of cell-specific characteristics, such as cell polarity, tight junction formation, mucus secretion and cilia formation [318]. Genetic and environmental factors likely have an effect on the tissue cultures and influence the inflammatory response, which is difficult to measure.

Whilst cell-modeling tools are useful, they do not accurately reflect the complexity of the airway environment and interpretation must take this into account.

5.1.1.3 Animal studies

Studies using xenografts have examined the inflammatory profile of the CF airway, using human fetal tracheal grafts in severe combined immunodeficiency (SCID) mice. These studies demonstrated that uninfected CF human fetal tracheal grafts have higher intraluminal concentrations of IL-8 at baseline and increased subepithelial leukocytes compared with non-CF grafts. Bacterial challenge with *PA* initiated intense damage to the mucosa in the CF airway graft

compared to the non-CF graft, leaving it vulnerable to further bacterial infection [319].

Murine models have demonstrated that following the instillation of PA-impregnated agar beads, CFTR-deficient mice have significantly higher concentrations of inflammatory mediators in BAL fluid, and greater mortality than controls, despite an identical infecting bacterial load [320]. Similar to *in vitro* models, not all murine models have shown this relationship, with some subsequent studies demonstrating similar levels in both groups [321]. More recently, studies in newborn CF-ferrets have indicated that defects exist in both innate immunity and inflammatory signaling. BAL obtained both immediately at birth and following first bacterial exposure during birth showed disturbances in inflammatory mediators, with higher TNF-α and IL-8 in CF ferrets compared to controls, and disturbance in cellular signaling pathways controlling immunity and inflammation (including the complement system and macrophage function) [322]. Studies in the CF pig have revealed that despite the fact that they develop spontaneous lung infections soon after birth, the levels of neutrophils a hypothesis theory that infection must be present to stimulate the inflammatory response; however the CF pig has not yet been explored to see if the response to infection is exaggerated [15].

5.1.1.4 Proposed mechanisms – leading to exaggerated inflammatory response

It has been proposed that defective CFTR alters several pathways that result in a dysregulated immune response. The mechanism of this is currently not known and there are several proposed hypotheses:

Epithelial cells with impaired CFTR function are hyperinflammatory.

- Increased activation of the NF-kB pathway; possibly as a result of abnormal CFTR function affecting ASL composition and volume, concentrating pro-inflammatory factors which leads to altered cell signaling and enhanced production of NF-kB. This factor translocates into the nucleus and increases the transcription of pro-inflammatory cytokine genes (including IL-8, TNF-α, IL-6 IL-1B), which ultimately increase airway neutrophilia In addition, impaired CFTR function results in hyper-polarised membrane potentials, which potentially leads to an increase in intracellular calcium ions that effect NF-kB signaling, particularly on exposure to pathogens [323].

- Defective CFTR affects fatty acid and cholesterol metabolism, resulting in a higher production of products that promote inflammation (arachidonic acid and leukotriene B_4) and decreased

levels of anti-inflammatory lipids [324].

- Abnormal intracellular ion transport caused by impaired CFTR function results in an accumulation of ceramide in the airways, inducing cellular apoptosis. There is subsequent deposition of DNA in the airways, which increases the adhesion of *PA* and promotes a downstream signaling cascade leading to chronic inflammation [325].

- Accumulation of mis-folded or unfolded CFTR protein in the endoplasmic reticulum (ER) in patients with CF, causes stress-induced cell death which results in an increase in inflammatory signaling pathways to repair tissue damage [300].

Epithelial cells with impaired CFTR function have impaired resolution of the immune response.

- There is a defect in lipoxin-mediated anti-inflammatory activity in the cystic fibrosis lung, with lipoxin concentrations in the airway fluid shown to be significantly lower in subjects with CF compared to other inflammatory lung conditions [304]. This is postulated to be due to reduced 15-lipoxygenase 2 expression; the gene that encodes for an enzyme involved in converting arachidonic acid into anti-inflammatory lipoxins [324].

- A study of murine alveolar macrophages showed that lack of CFTR function resulted in abnormal TLR4 localisation, which resulted in an increase in pro-inflammatory pathways (modulated by increased NF-kB) and a decrease in the degradation of the TLR which affects the down-regulation of the pro-inflammatory state [326].

- It has been proposed that interferon (IFN) signalling is blunted, which interferes with eradication of bacteria, and increases susceptibility to viral infection [326].

Currently there is inconclusive evidence to determine whether CF airways generate excessive inflammation for a given bacterial load, and whether the airway epithelium dysregulates pathways thus contributing to the excessive inflammation which characterises CF lung disease. Alternative models for exploring the role of CFTR function in regulating the inflammatory response are required.

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5.1.2 Previous work performed in the Departments of Gene Therapy and Paediatric Respiratory Medicine

This study compared the inflammatory phenotype of nasal epithelial cells from subjects with CF with those from patients with PCD. PCD shares a similar pathology of impaired MCC and airway neutrophilia to CF, but cells have no genetic CFTR defect, and presumed normal CFTR protein function. Comparison of the two cell types potentially allows differentiation between the secondary effects of chronic infection and inflammation on MCC and epithelial cells with that of specific CFTR dysfunction. The hypothesis for this study was that the cells from subjects with CF, with absent CFTR function, would produce higher levels of pro-inflammatory cytokines following bacterial stimulation than those from subjects with PCD or healthy controls.

Epithelial cells were obtained by nasal brushing samples from patients with CF and PCD who were attending respiratory clinic at the RBH and age/sex matched healthy controls. The samples were infected with known concentrations of the common respiratory bacteria *SA, PA* or *HI* and supernatant surrounding the cells was removed at 1 and 24 hours and later analysed using IL-8 Human ELISA Kit (Life Technologies) to quantify the level of this pro-inflammatory cytokine. This ELISA was also used in the pilot studies for the main experiment and the methodology is described in Section 2.2.5.1.

Results in 38 subjects, CF (10), PCD (10) and controls (18) surprisingly demonstrated that the levels of IL-8 in the supernatant from cells from subjects with PCD cell were significantly lower ($P<0.05$) at 24 hours than CF and controls, which were similar (Figure 5.1-1)

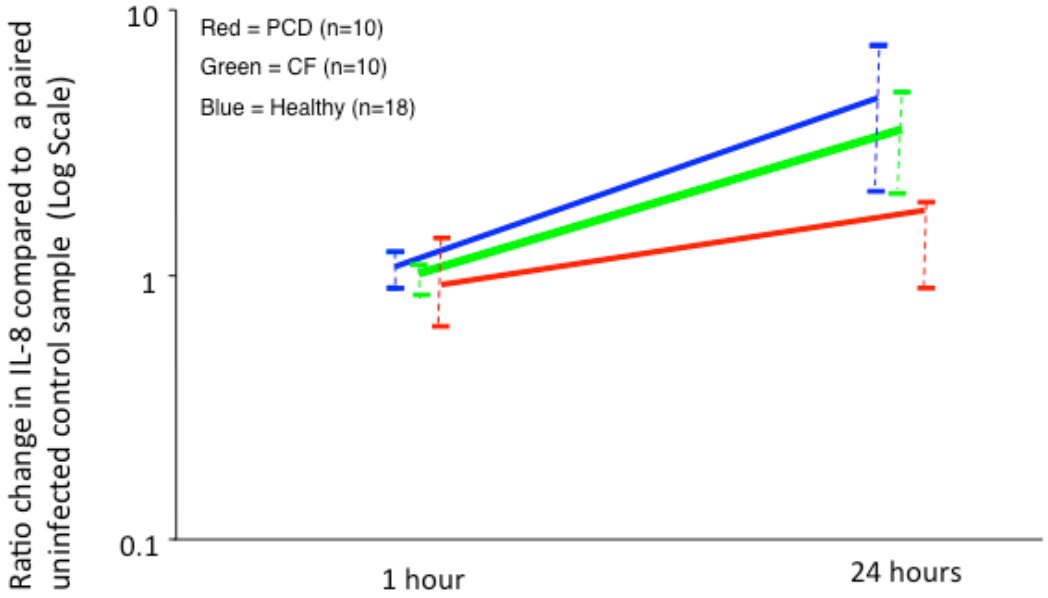


Figure 5.1-1: Inflammatory response (IL-8) of nasal epithelial cells in suspension infected with *PA* demonstrating a smaller inflammatory response in patients with PCD than CF or controls at 24 hours ($p<0.05$). *Data presented are mean (pg/ml) and range of values for each subject group at 1 hour and 24 hours after exposure to PA*

These results did not support the hypothesis that cells with reduced/absent CFTR function produce an exaggerated pro-inflammatory response to infection; the epithelial cells from subjects with PCD appeared to mount a blunted inflammatory response when exposed to bacterial stimuli, opposite to that seen *in-vivo* [327]. It is likely that these unexpected results were due to limitations of the cell suspension model; discussed above.

The study described in this Chapter is a development of the pilot study in which cells cultured at an air-liquid interface (ALI) were chosen to test the hypothesis, as they better replicate *in vivo* conditions than cells in suspension.

5.1.2.1 Cells cultured to an ALI

Cells obtained from subjects by brushings, are cultivated in a controlled environment (described below in detail). The result is a confluent cell monolayer where medium is only supplied to the basal chamber and the apical surface of the cells is exposed to air, which mimics the conditions found in the airway. Cells can either be cultured to this 'basal' level, or can be further cultivated to allow cells to differentiate towards a ciliated epithelial phenotype that resembles the human respiratory epithelium *in vivo*. These cells have normally motile cilia, secrete mucus, and develop

stable transepithelial electrical resistance thus providing a robust experimental tool. For experiments that use *in vitro* models to explore cell response to bacterial stimulation, ALIs have the advantage that it is possible to apply bacteria only to the apical surface as would occur naturally in the airway. The disadvantage of allowing final stage for ciliogenesis to occur is that this stage of cell culture takes approximately 21 days and is frequently unsuccessful due to failure of the cells to propagate. This lengthens the time scale of studies and increases the risk of cell infection thus reducing the yield of successful cell cultures. It is unknown whether cells cultured to the basal level produce a comparable inflammatory response to bacterial stimulation as ciliated cells and whether these can be used as a surrogate tool.

This present study was designed to enhance understanding of the relationship between CFTR function and the response to bacterial challenge of nasal epithelial cells, utilising cells cultured at an ALI as a better representative of *in vivo* conditions than cells in suspension.

The hypothesis was that cells from subjects with CF, with absent CFTR function, would produce higher levels of pro-inflammatory cytokines following bacterial stimulation than those with PCD or healthy controls.

5.1.3 Study aims:

- To define the relationship between CFTR function and inflammation by comparing the inflammatory response to bacterial challenge of CF nasal epithelial cells cultured at an ALI to those of wild-type and non CF CSLD, in this case PCD

Objectives

- To culture cells at an air-liquid interface from subjects with CF, PCD and controls and expose the cell cultures to common respiratory bacteria
- To collect supernatant from the cell cultures at regular time intervals (1, 4, 24 and 72 hours) following bacterial stimulation, and measure the quantity of pro-inflammatory cytokines in the supernatant
- To compare the inflammatory profile of the supernatant obtained from CF, PCD and controls at all time points

This study was designed and performed by myself assisted by Dr Amelia Shoemark, Senior Scientist, RBH, London. All of the data analysis and statistics were performed by myself.

5.2 Methods

5.2.1 Overview of study design

Nasal brushings were obtained from subjects with CF, PCD and healthy volunteers. These cells were cultured at an ALI interface to a ciliated cell phenotype until 4 wells from the same subject had differentiated. These were infected with either one of the common respiratory disease causing organisms; *SA, PA, HI* or phosphate buffered saline (PBS) (control). After 1 hour, the organisms were killed with local application of antibiotics (streptomycin, penicillin and gentamicin) and supernatant from all of the samples was collected at 1 hour, 4 hours, 24 hours and 72 hours following the initial infection. The supernatant was frozen at -80^{o}C, and later analysed using the MSD platform, designed to detect the presence and quantity of inflammatory mediators in tissue supernatant. The inflammatory response elicited from each group to each bacterial stimulus was compared.

5.2.2 Pilot study

Prior to commencing the study described above, it was necessary to establish the experimental parameters. This included:

- The most appropriate cell lineage:
  - To determine whether cells cultured to the basal stage elicit the same inflammatory response as the fully ciliated phase, and therefore if basal cells could be used as a quick surrogate for planning the main study.
- The optimal bacterial concentration:
  - To be both sufficient to stimulate a cytokine response detectable by standard laboratory assays, and also be successfully killed by antibiotics to ensure that the period of bacterial exposure to the cells could be controlled.

These matters were addressed in a pilot study, consisting of 3 experiments described below in section 5.2.4.

5.2.3 Laboratory methodology

The following section describes the elements of the methodology common to both the pilot and main experiment, based on approved departmental protocols.

5.2.3.1 Nasal brush biopsies

Nasal brushings were obtained as described in Section 2.2.4.

5.2.3.2 Cell culture

Airway Liquid Interface (ALI) cell cultures were grown as according to the RBH NHS Trust Electron Microscopy Unit - PCD diagnostic service standard operating procedure.

Equipment

Recirculating class II safety cabinet with HEPA filter
Vacuum pump
37ºC CO_2 Incubator with sealable door and HEPA filter
Laboratory fridge freezer
Nikon Eclipse TI inverted microscope with 40x lens
Small incubator (for warming media)
Balance (sensitive to 0.01g)
A list of the reagents, including calibration material is included in Appendix A.

Culturing basal cells:

The cytology brush was agitated in 1ml of BEGM until all the material was removed and the BEGM was transferred to a collagen coated well (12 well plate). The cell cultures were incubated at 37^{o}C, 5% CO_2. Every 2-3 days the BEGM was changed and the confluence of the basal cell layer visualised under the microscope and documented. When 80-90% confluence had been reached the cells were expanded into a flask.

Flasks:

To expand the wells into a tissue culture flask (T25), the BEGM was removed from the well using a vacuum pump. 1ml of Hanks Balanced Salt Solution with calcium and magnesium (HbSS-Ca-Mg) was added and incubated for 5 minutes at 37^{o}C, it was then removed and 0.5ml of Trypsin/ EDTA added to the well and incubated for 3 minutes. Both of these steps facilitate detaching the cells from the well. The cells were gently pipetted off the trypsin and transferred into 10ml BEGM in a 15ml tube. This was spun at 1100rpm at 19^{o}C for 5 minutes, the supernatant removed, the pellet resuspended in 1 ml BEGM and added to a collagen coated T25 flask containing 4ml BEGM. Flasks were fed every 2-3 days and confluency assessed. When 80-90% confluence was attained (at least 4 days) it was split into transwell supports.

Transwell supports:

A 12 well plate with 4 transwell inserts was placed in the fume hood, exposed to UV light. 5ml of HbSS Ca-Mg was added to the T25 flask for 5 minutes (37^{o}C) which was replaced by 1.5ml of Trypsin/EDTA, incubated for 3 minutes, and then transferred into a 15 ml tube with 10ml BEGM. This was centrifuged at 1100 rpm for 5 minutes at 10^{o}C. Supernatant was removed and the pellet resuspended in Air Liquid Interface (ALI) media to make 330,000 cells/ml. 0.25ml (roughly 83,250 cells) were transferred into the wells on the plate (usually 4). 1 ml of ALI media was added to the basolateral side of each well. Every 2-3 days the apical liquid and basolateral liquid were replaced with 250µl and 1 ml of ALI media respectively. The change in colour of the media was noted, and 2 feeds after the media changed from pink to orange (on average 7-10 days) they were transferred to air liquid interface (ALI).

ALI cultures:

When 100% confluence was achieved, the apical fluid was removed using the vacuum pump and the basolateral fluid replaced with 700µl ALI media. This was repeated every 2-3 days. The colour of the media was noted as change from pink to orange indicated cells were successfully metabolising ALI media. Cilia start to emerge from day 12 of ALI onwards and are visible under the microscope at day 21 of ALI.

5.2.3.3 Common problems encountered

Infection: Cells became infected either from the original source or contamination from apparatus. To try and avoid this, penicillin, streptomycin, gentamicin and amphotericin were added to the media (BEGM and ALI). When wells became infected they were removed immediately and disposed of in Virkon to prevent cross contamination.

Failure to propagate: A minimum number of 82,000 cells are required for basal cells to propagate on the 12 well plate. Insufficient numbers meant that samples failed to develop for splitting into flasks.

Failure to ciliate: Samples can fail to ciliate for several reasons. Either a mixed cell population can develop despite the use of selective growth media, or they appear to grow 'tired' during the process and fail to successfully ciliate.

5.2.3.4 Bacteria

Growth of bacteria

Bacteria known to be a common cause of infection in patients with CF and PCD were considered for the study. *PA SA and HI* were chosen as they are the most common infecting organisms [328].

The bacteria (*PA*: PA01 lab strain, and *HI* and *SA*: clinical strains) were stored at -20^{o}C on beads. 4 days prior to experimental use, 2 beads were placed in 10ml of Tryptone Soya Broth (TSB) and agitated overnight at 37^{o}C. Following this, each strain was subcultured 3 times on appropriate agar: *SA* and *PA* on Colombian horse agar and *HI* on chocolate agar. 24 hours prior to the experiment, a single colony of each bacteria was used to inoculate a separate container of 10mls of TSB. These were cultured overnight and agitated at 37^{o}C to provide the bacterial culture.

Concentration of bacteria

The concentration of bacteria was measured using optical density at a wavelength of 600nm (OD_{600}). OD is measured in a photometer where light is emitted by a source and detected and quantified on a photoelectric cell. A sample of the bacterial culture is placed between the light source and the cell in a cuvette, causing the light to scatter, so that the electrical signal is weaker

than with a cell-free cuvette. The weaker electric signal is converted to an extinction or OD value, which can be used to estimate the concentration of the bacteria, as illustrated in Figure 5.2-1.

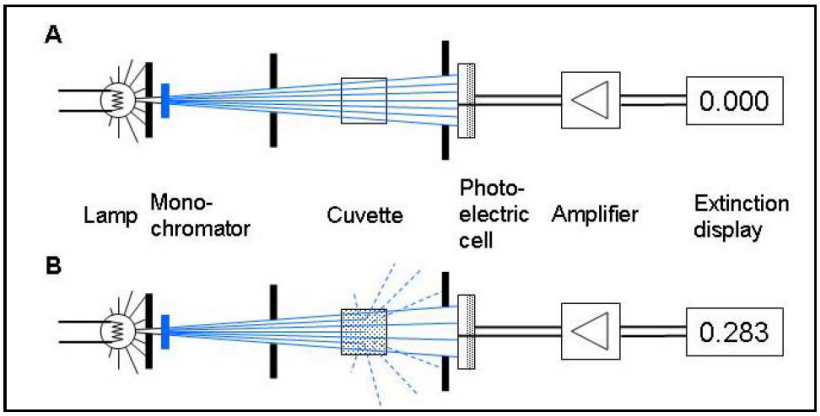


Figure 5.2-1 Typical setup of an absorbance measurement in a photometer, illustrating a) a control sample with a cell-free cuvette, and b) bacteria in the cuvette, causing the light to scatter resulting in a weaker electrical signal

Method: 2 ml of each bacterial solution suspended in TSB was spun at 3000g for 10 minutes at 4°C to retrieve a pellet. The pellet was re-suspended in PBS and the OD_{600} determined using UNICAM UVI (ultraviolet visible instrument). The OD_{600} was adjusted to the required OD_{600} by addition of PBS. It is known from the literature that an OD_{600} of 0.1 equates to approximately 1 x 10^8 cfu/ml of *PA,* however the OD required to obtain approximately the same concentration of cfu/ml of *SA* and *HI* is not as well established. This was addressed in the pilot study, Experiment 2 (5.2.4.2).

5.2.4 Pilot Study – methods

5.2.4.1 Experiment 1

This experiment was performed to determine whether cells cultured to the basal stage elicit the same inflammatory response as the fully ciliated phase, and therefore if basal cells could be used as a surrogate for fully ciliated ALIs.

Method

5 sets of cell cultures were selected for this experiment, selected from samples that were initially grown for clinical purposes but subsequently found not to have a diagnosis of CF or PCD and no

longer required. 3 sets of the cell cultures contained fully differentiated ciliated cells, and 2 consisted of predominantly basal cells (determined by visual inspection). These were derived from 4 different donor subjects; with the 3 sets of ciliated cells from 3 different donors, and the basal cells both from the same, 4th donor, due to the availability of cell cultures available. 24 hours prior to the experiment, all cells were fed with ALI media which had been made without the normal addition of antibiotics or steroids, to prevent any artificial suppression of the inflammatory response.

The bacteria were prepared as described above. *PA/SA* and *HI* were reconstituted in PBS and the concentration adjusted to produce concentrations of each at both OD_{600} =1, and OD_{600} = 0.1.

Start

The cells were infected with 200µl of bacteria or PBS (control). Not all sets of cell cultures had 4 available wells therefore infection of the cells was adjusted as illustrated in

Table 5.2-1.

Cell Number	Cell type	Well 1		Well 2		Well 3		Well 4
		Bacteria	Conc OD_{600}	Bacteria	Conc OD_{600}	Bacteria	Conc OD_{600}	CONTROL
1	Ciliated	HI	1	SA	1	PA	1	PBS
2	Ciliated	HI	0.1	SA	0.1	X	n/a	PBS
3	Ciliated	X	n/a	X	n/a	PA	0.1	PBS
4	Basal	HI	1	SA	1	PA	1	PBS
5	Basal	HI	0.1	SA	0.1	PA	0.1	PBS

Table 5.2-1: Layout of the wells, cell type and bacteria in each group of cell cultures

1 hour

200µl of supernatant was sampled from the apical region, divided into 4 x 50µl cryovials and frozen at -80^{o}C. 700µl of supernatant from the basolateral region was frozen in a cryovial at -80^{o}C. 700µl of ALI media without steroids, but with antibiotics, was added to the basolateral region of all the cells. 200µl of ALI media with the addition of antibiotics was added to the apical section of the

basal cells. 200µl of PBS containing antibiotics was added to the apical section of the ciliated cells. All cells were placed in the incubator at 37^{o}C.

4 hours

Supernatant was collected and stored as at 1hour. 10µl of supernatant from each well infected with bacteria, was removed and plated onto the appropriate agar and incubated overnight at 37oC. These were examined at 24 hours to look for evidence of bacterial growth.

24 hours

Supernatant was collected and stored as before. 10µl of supernatant was only removed from the samples in which bacterial growth had been documented at 4 hours. These were incubated over night and examined at 24 hours to look for evidence of bacterial growth.

72 hours

Supernatant was collected and stored as before. 10µl of supernatant was only removed from the samples in which bacterial growth had been documented at 24 hours. These were incubated over night and examined at 24 hours to look for evidence of bacterial growth. The cells were examined under the (Axiovert inverted microscope, Zeiss) microscope to observe cellular integrity and ciliary beat frequency as a marker of cell damage.
At a later date, the samples of supernatant were thawed and analysed using an IL-8 Human ELISA Kit (Life Technologies) following the manufacturers instructions (Section 2.2.5.1). The standard curve range, 15.6-1000ρg/mL for this ELISA is comparable to the dynamic range of IL-8 detection on the MSD platform, 0.12-10000 ρg/mL (sensitivity >0.12 ρg/mL), which will ultimately be used in the main experiment.

Results

- Cell lineage and sample site
    - Basal cells did not produce an inflammatory response detectable within the standard ELISA range.
    - Levels of IL-8 measured in the supernatant sampled from the basolateral segments were significantly lower than that from the apical segments.
- Bacterial concentration

- Elevated IL-8 levels were detected in the apical supernatant of ciliated cells following infection by all bacteria at both concentrations (OD_{600} =0.1 and OD_{600} =1) as illustrated in Figure 5.2-2, however the response produced following stimulation by *PA* was greater than that produced by *HI* and *SA*

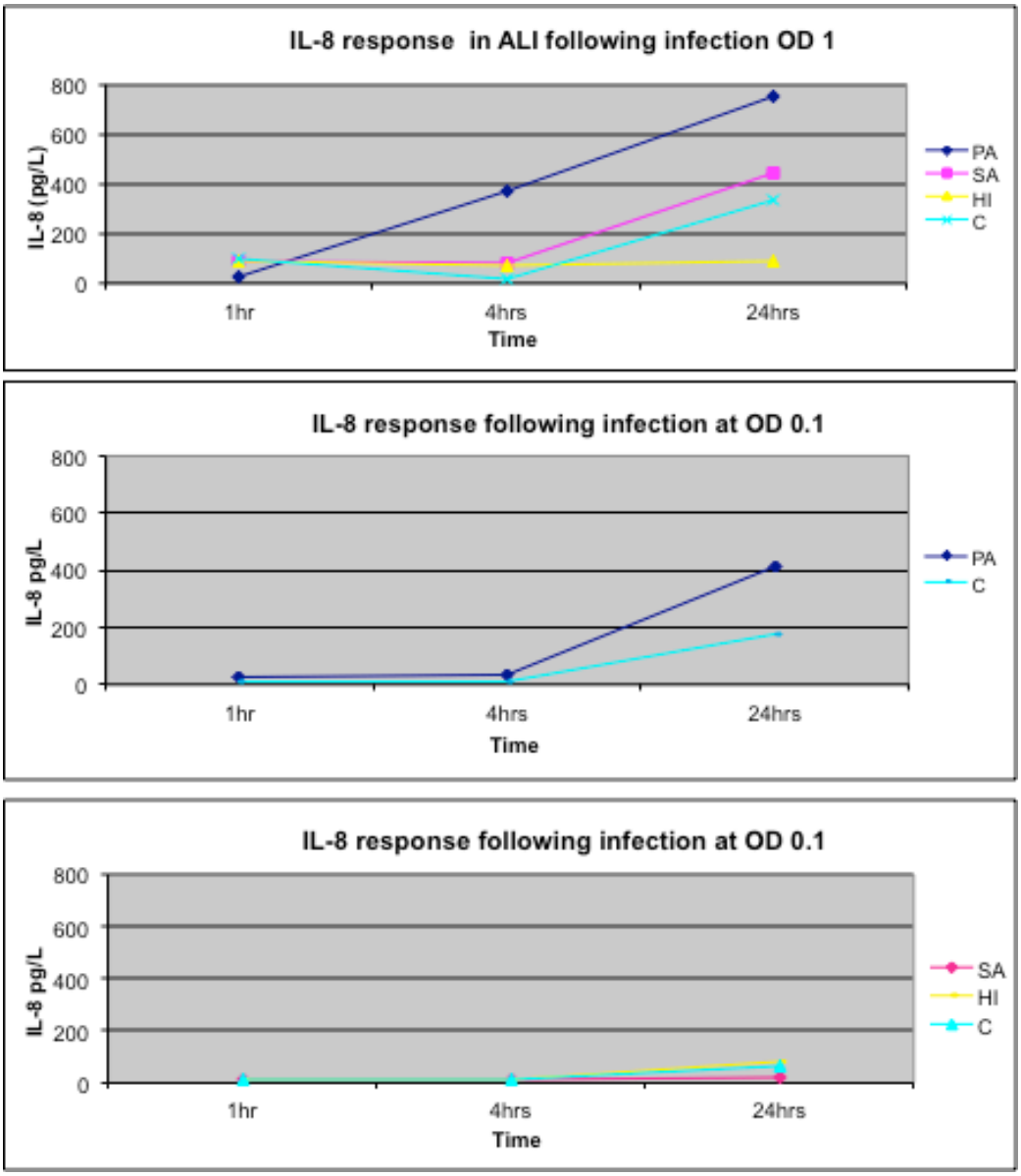


Figure 5.2-2: IL-8 response following infection with a) higher concentration of the all bacteria OD_{600} =1 *PA, SA, HI* and control, and b) a lower concentration of *PA* only (OD_{600} =0.1 *PA*) and control and c) a lower concentration of *SA* and *HI* (OD_{600} =0.1 *SA/ HI*) and control

- Following infection with all of the bacteria at the lower concentration (OD_{600} 0.1), cilia were visualised to be beating in the well and no holes in the monolayer or visible damage to the

cells had occurred at 72 hours. Infection at the higher concentration of bacteria (OD_{600}=1) showed more cilial damage, and dead cells had detached and were floating in the apical media at 72 hours.

- No growth of either *PA, HI* or *SA* were visualised on the agar plates at 1,4,24 or 72 hour points at either concentration

Discussion

Cells cultured to the basal stage did not produce a sufficient inflammatory response to be detectable within the limits of the IL-8 ELISA kit (15.6-1000pg/ml). The ciliated cell cultures showed a rise in levels of IL-8 following stimulation with *PA* at bacterial concentrations OD_{600}=0.1 and OD_{600}= 1, therefore these fully differentiated ciliated cultures were selected for all subsequent experiments. At both concentrations there was no bacterial growth on the agar plates following antibiotics, indicating successful bacterial killing. As there was significant evidence of cell damage following inoculation where OD_{600} =1, a lower concentration was considered preferable. A value of OD_{600}=0.5 was chosen for subsequent experiments, to maximise the inflammatory response and limit cell damage.

There was less of an increase in IL-8 following bacterial stimulation by *HI* and *SA* compared to when the cells were infected with *PA.* In the methodology, we had reconstituted each bacteria to either OD = 0.1 or OD = 1, however this did not account for the different cell density of the bacteria. It is known that for *PA* OD_{600}=0.1 contains approximately $1x10^8$ cfu/ml (reference), however this has not been established for *HI* and *SA*. As *HI* and *SA* are larger than *PA*, it is likely that for a similar $OD_{600.}$there are less cfu/ml, which may explain the reduced inflammatory reaction response to these 2 bacteria. Alternatively, *PA* may induce a different inflammatory profile to the other 2 bacteria. An experiment was required to determine the OD_{600} for each strain required to acquire the same number of cfu/ml for each bacteria.

5.2.4.2 Experiment 2

This was performed to determine the OD of *HI* and *SA*, and compare to the known OD of *PA.* Bacteria cultures were prepared as described (5.2.3.4). 1ml of each bacterial broth suspension was spun at 3300 rv/minute for 5 minutes to retrieve a pellet, which was re-suspended in 1ml of PBS. The OD_{600} of this bacterial suspension was measured, and adjusted by the addition of PBS to obtain a 1ml sample of each bacteria where OD_{600}=0.1. This 1ml sample was distributed in 8

aliquots, with 100µl added to 900µl of PBS in the first aliquot and subsequent serial dilution. 2 x 10µl from each aliquot were applied to designated sections of the appropriate agar plate for each bacteria, and the plates were incubated at 37^{o}C. At 24 hours, the numbers of cfu grown for both samples at each dilution for all of the bacteria were counted.

Results

Table 5.2-2 documents the results for the number of cfu grown for each bacteria at each serial dilution. This demonstrates that the OD for *HI* is approximately 10 logarithms higher than *PA*, and *SA* 10 logarithms higher again. These results replicate the published studies which have shown that OD_{600} =0.1 for *PA* contains approximately $1x10^{8}$ cfu/ml.

Dilution	PA 1	PA 2	HI 1	HI 2	SA 1	SA 2
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	137	118
4	+	+	104	120	13	17
5	80	+	17	13	2	3
6	38	10	8	2	0	0
7	1	2	3	1	0	0
8	2	0	1	0	1	0

Table 5.2-2- Number of cfu grown for each bacteria (*PA, HI* and *SA*) at each serial dilution

Discussion

To obtain a similar number of cfu of *HI* and *SA* to *PA*, x10 and x100 the initial volume of these species is required respectively compared to *PA.* This can be spun down to retrieve the pellets, which could then be reconstituted in the same volume (200µl) of PBS to produce a comparable cfu/ml. It would not be possible to measure the OD of these bacteria at this concentration as it would lie above the optimal range (OD >0.8 leads to less accurate results). Instead, for future experiments, the amount of bacteria required to make a 1ml suspension with an OD of 0.1 was calculated, then the volume of bacterial solution taken was scaled up accordingly.

5.2.4.3 Experiment 3

It was necessary to determine the most appropriate way to collect the supernatant. In experiment 1 the cells had been covered with PBS for the 72 hour duration of the experiment to maximise the collection the inflammatory mediators, however cells cultured at ALI rely on the apical surface of the cells being exposed to air to replicate *in vivo* conditions.

Experiment 3 was performed to assess 2 issues:

- Whether leaving the cells uncovered from the 4 hours onwards (allowing for bacterial killing with antibiotics from 1 hour to 4 hours), and adding 200µl of PBS for 10 minutes prior to sample collection would collect comparable inflammatory mediators to the covered cells.
- Whether the 3 species, with the OD_{600} adjusted to provide the same number of cfu/ml, would produce a comparable inflammatory profile detectable by standard lab assays.

Method

2 cell cultures were utilised for this experiment, again initially grown for clinical purposes but subsequently found not to have a diagnosis of either CF or PCD. Bacteria were prepared as described in Section 5.2.3.4 and the OD_{600} adjusted to ensure that each sample had equivalent to $5x10^8$ cfu/ml. 3 wells from both cell cultures were infected with 200µl of each of bacteria and 1 with 200µl of PBS (control).

This experiment was performed as described for Experiment 1 with the following exceptions. At 4 hours, following the collection of the supernatant, 200µl of PBS with antibiotics was added to the apical surface of sample 1, and sample 2 was left uncovered. At the subsequent time points (24 and 72 hours) 200µl of PBS were added to the apical section of sample 2, and agitated for 10 minutes prior to collecting and storing the sample.

Results

- Uncovered cells vs covered cells
  - IL-8 detectable levels at these concentrations comparable to those in experiment 1
  - A comparable level of IL-8 was detected following bacterial infection with all 3 bacteria.

- *SA* colonies grew on plate for Sample 1 at 4 and 24 hours.

Discussion

ALI cells left uncovered yielded similar quantities of inflammatory mediator to the samples that had remained covered between experimental time points. As uncovered conditions better replicates *in vivo* conditions, this method was carried forward to the main experiment. When the concentrations of the bacterial suspensions were adjusted to provide a similar concentration of cfu/ml, comparable levels of IL-8 were obtained with different organisms. *SA* colonies were visualised on the agar plate at the 4 and 24 hour time points, indicating microbial killing by the antibiotics was unsuccessful. The decision was made to reduce the concentration of *SA* to half of that previously used.

Having established the optimal cell lineage, conditions, sampling sites and bacteria concentration from the pilot study, we went on to perform the main study, described below in detail. For this study, the MSD platform was chosen to analyse the concentration of inflammatory cytokines, as compared to standard ELISA. The MSD platform offers an increased dynamic range (for IL-8: 0.17-10000 pg/mL compared to 15.6–1,000 pg/mL respectively), improved sensitivity, and reduced sample and reagent requirements. Furthermore, it provides the opportunity to measure multiple cytokines simultaneously.

5.3 Main experiment – methods

An overview of the experiment is illustrated in Figure 5.3-1. Nasal brushings were performed (section 2.2.4) on subjects with PCD, CF and healthy controls. Respiratory ciliated cells were cultured at ALI as previously described (Section 5.2.3.2). In total 51 subjects were recruited to participate in the study. Of these, 13 (25.5%) sets of ALI cell culture were successfully cultivated to produce 4 wells of ciliated epithelial cells: healthy controls (5), CF (4), and PCD (4). All CF subjects were F508del homozygous. The subjects with PCD all had ultra-structural defects associated with PCD; 2 had outer dynein arm defect (ODA) and 1 outer and inner dynein arm defect (IDA), 1 a transposition defect.

To ensure that the cells were a similar age and to account for both the time period involved in collecting samples, and the ALIs reaching ciliated stage at different times, the experiment was

performed in 3 stages. Each stage utilised cells from either the CF or PCD group, and included a healthy control sample. 24 hours prior to the experiment, all cells were fed with ALI media which had not had antibiotics or steroids added to prevent any artificial suppression of the inflammatory response.

Bacteria were prepared as described in section 5.2.3.4 to produce samples containing $5x10^8$ cfu/ml of *PA* and *HI* and $2.5x10^8$ cfu/ml of *SA*.

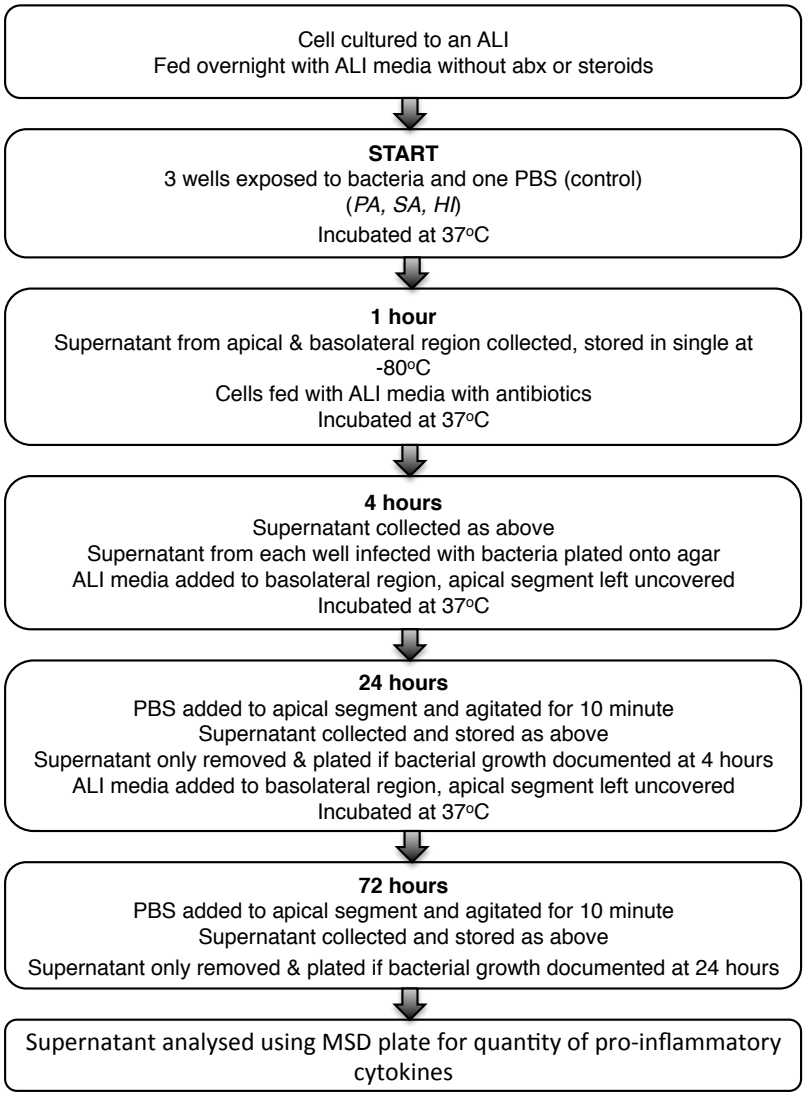


Figure 5.3-1: An over view of the main experiment comparing the inflammatory response to bacterial challenge of CF nasal epithelial cells cultured at an ALI to those from patients with PCD and controls

Start

The cells were infected with either 200µl of bacteria or PBS (control) applied to the apical cell membrane.

1 hour

200µl of supernatant was sampled from the apical region, divided into 4 x 50µl cryovials and frozen at -80^{o}C. 700µl of fluid from the basolateral region was frozen at -80^{o}C in a cryovial. 700µl of ALI media without steroids, but with antibiotics, was added to the basolateral region, and 200µl added to the apical section of the cells. All cells were incubated at 37^{o}C.

4 hours

Supernatant was collected and stored as before. 10µl of supernatant from each well infected with bacteria, was removed and plated onto the appropriate agar. Plates were incubated overnight at 37^{o}C and examined at 24 hours to look for evidence of bacterial growth. 700µl of ALI media without steroids, but with antibiotics, was added to the basolateral region. The apical sections were left uncovered.

24 hours

200µl of PBS were added to the apical section of the wells and agitated for 10 minutes. Supernatant was collected and stored as before. 10µl of supernatant was only removed from the samples in which bacterial growth had been documented at 4 hours.

72 hours

200µl of PBS were added to the apical section of the wells and agitated for 10 minutes. Supernatant was collected and stored as before. 10µl of supernatant was only removed from the samples in which bacterial growth had been documented at 24 hours.

5.3.1 Sample analysis

The samples were thawed and the cytokine profile measured using a Human ProInflammatory 9-plate Tissue Culture Kit (Meso Scale Discovery (MSD) platform) (K15007B-1), designed to quantify the presence of GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, and TNF-α in tissue culture supernatant. The methodology for this was described in detail in Section 2.2.5.2. 2 of the samples were run twice on the MSD platform to assess the repeatability of the results.

As in the previous studies (both the cell suspension and the pilot experiments), IL-8 analysis had been performed using a Human ELISA Kit (Life Technologies) (as previously described), 4 samples from the cell suspension experiment were also analysed on the MSD plate; 2 of which were reported to have low levels of IL-8 and 2 which had high levels. This was performed to see if both analysis platforms produced similar results, thus allowing the results from both experiments to be compared.

5.3.2 Data analysis

The results were analysed using Prism 6 software (GraphPad Software Inc, CA, USA). As data were non-parametric, the Kruskal-Wallis test was used to compare the 3 patient groups at each time point, with *post hoc* Dunn's comparison test for multiple statistical comparisons. The null hypothesis was rejected at $p<0.05$.

5.4 Results

5.4.1 Assessment of successful bacterial killing

Bacterial growth was only detected on 3 plates in total. 2 of these were *SA* at 24 hours and at 72 hours from the same cell culture, and the remaining 1 was *SA* from another cell culture at 24 hours, however this was not grown again at the 72 hour point.

5.4.2 Inflammatory profile in supernatant

Data from all cytokines were analysed, however the results displayed below focus on IL-6, IL-8, TNF-α and IFN-γ as they are considered to be the most clinically relevant inflammatory mediators (ref) and the others did not display a significant bacterial response.

5.4.2.1 Baseline cytokine levels

To determine whether there was a difference in baseline cytokines between the 3 groups, the level of the cytokines IL-6, IL-8, TNF-α and IFN-γ detected following 1 hour exposure to PBS (control) were compared. The results are illustrated in Figure 5.4-1

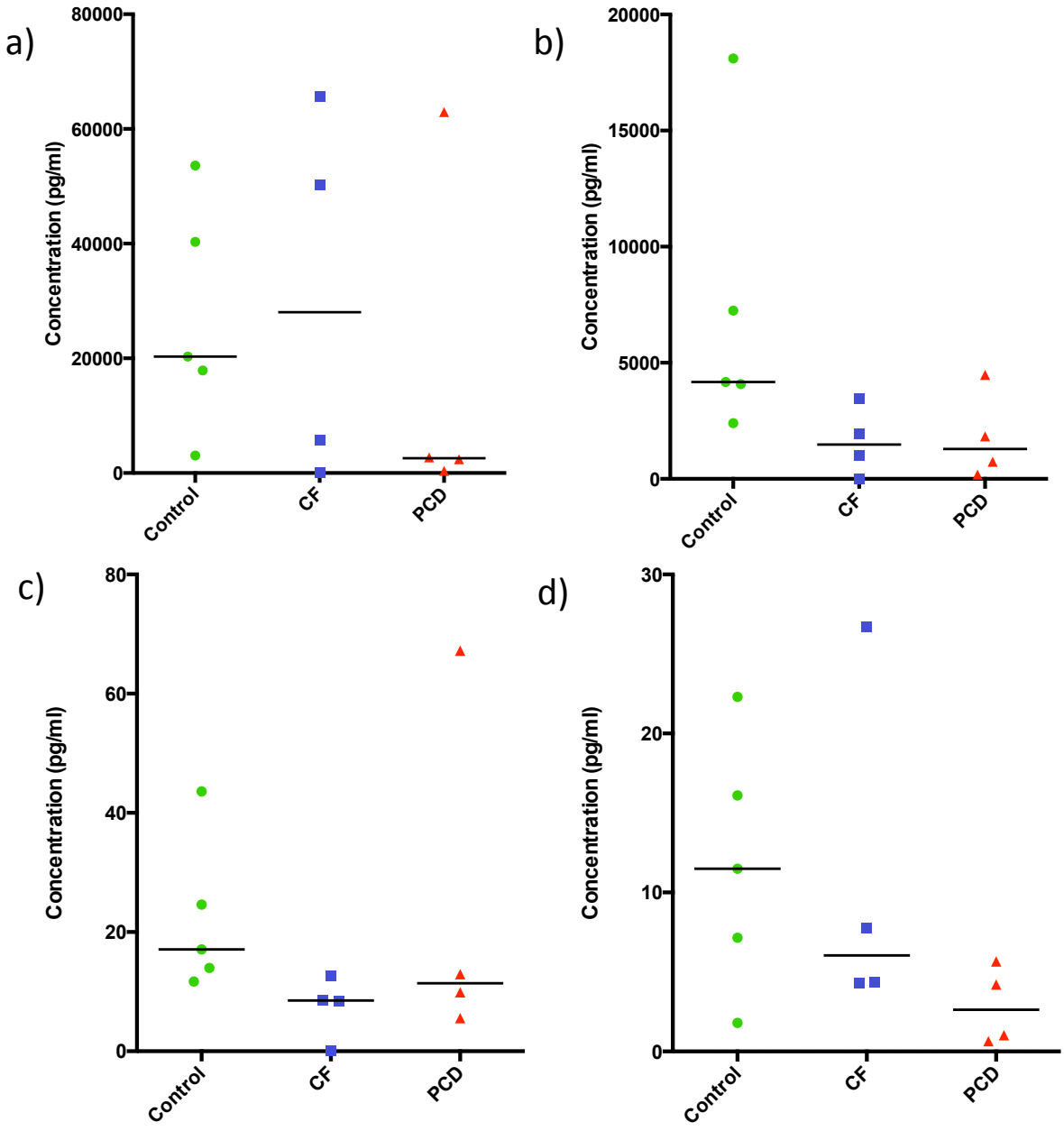


Figure 5.4-1: Supernatant cytokine profile at baseline. *a) IL-6 b) IL-8 c)TNF-α d) IFN-γ.* Cytokine concentrations are illustrated as pg/ml with data from each patient expressed as a single data point. There was no statistical difference in the levels between the control groups, CF and PCD. *The horizontal line indicates the median value of the group.*

Comparison of the patient groups using the Kruskal-Wallis test demonstrated no statistical difference in baseline levels of the cytokines (Figure 5.4-1). This was replicated with all other cytokines measured on the MSD panel

5.4.2.2 Level of cytokines following bacterial stimulation

Following this, the level of cytokines detected at 1, 4, 24 and 72 hours in each patient group following bacterial stimulation with *HI, PA* and *SA* were compared. The level of cytokine measured is displayed as a ratio to the control group, where the cells from the same subject were exposed to only PBS. The results are illustrated in Figure 5.4-2 - Figure 5.4-5. For each set of graphs, the time (x axis) represents the period of time (hours) after cells had been exposed to the bacteria (or control) before the supernatant was collected. The y axis measures the level of cytokine detected when a) the cells were unstimulated (control cells exposed only to PBS), and in response to bacterial stimulation by b) *HI* c) *PA and* d) *SA.* The values for each group are expressed as median and IQR.

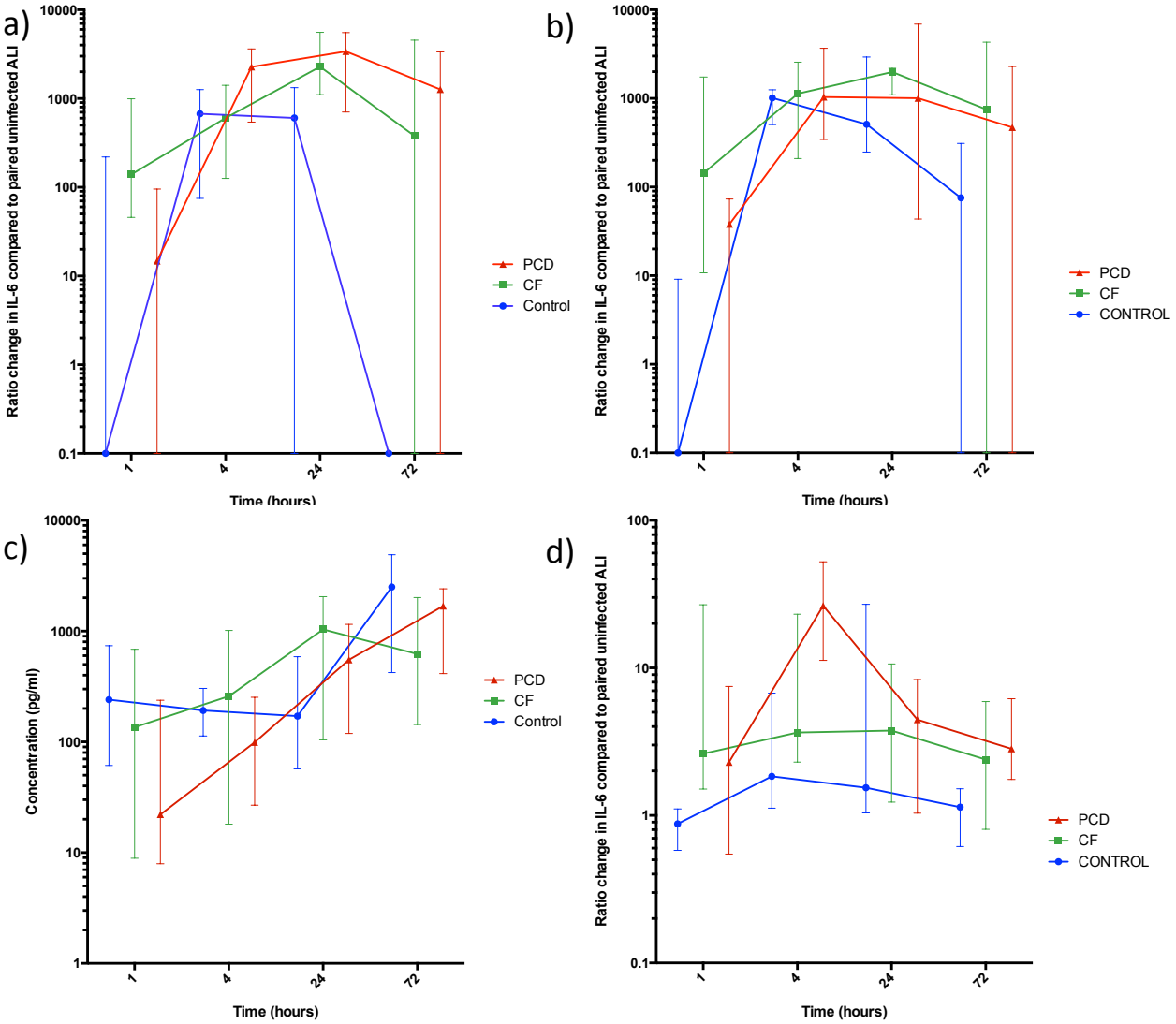


Figure 5.4-2: Levels of IL-6 (pg/ml)

Figure 5.4-2 illustrates that the cells mounted an inflammatory response, measured by levels of IL-6 increasing 2-3 fold, in all 3 patient groups to *HI and PA* and to a much lesser extent *SA* (NB: scale only to 100 compared to 10,000) at the 4 hour and 24 hour time points. The response detected at 72 hours was more variable. Comparison of the levels of IL-6 using the Kruskal-Wallis test, and Dunn's multiple comparison test demonstrated no significant difference between the levels detected between any of the patient groups, at any time points.

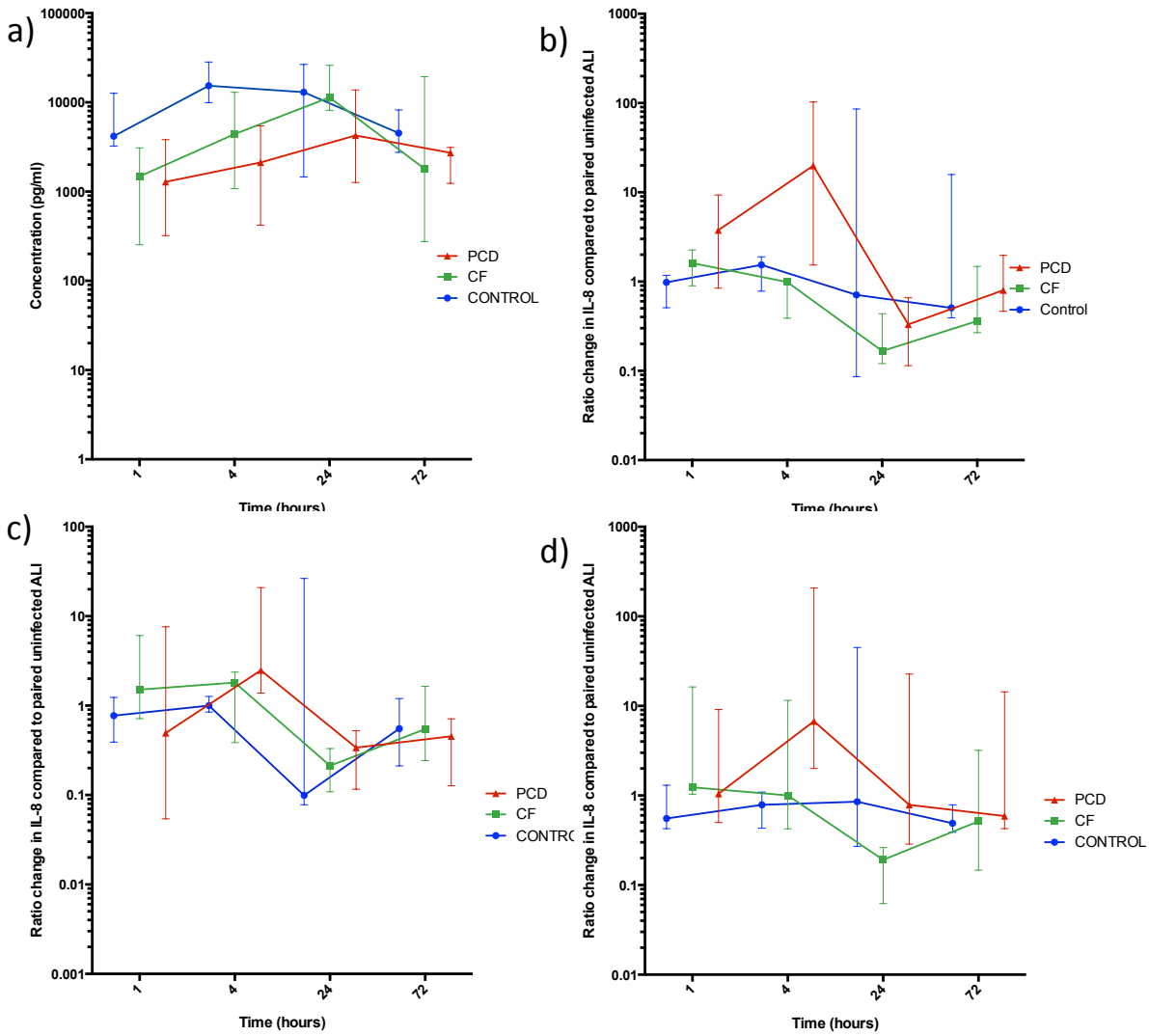


Figure 5.4-3: Levels of IL-8 (pg/ml)

Figure 5.4-3 shows no consistent rise in IL-8 was detected following exposure to any of the pathogens, which did not replicate the findings from the pilot study, in which an IL-8 response was demonstrated.

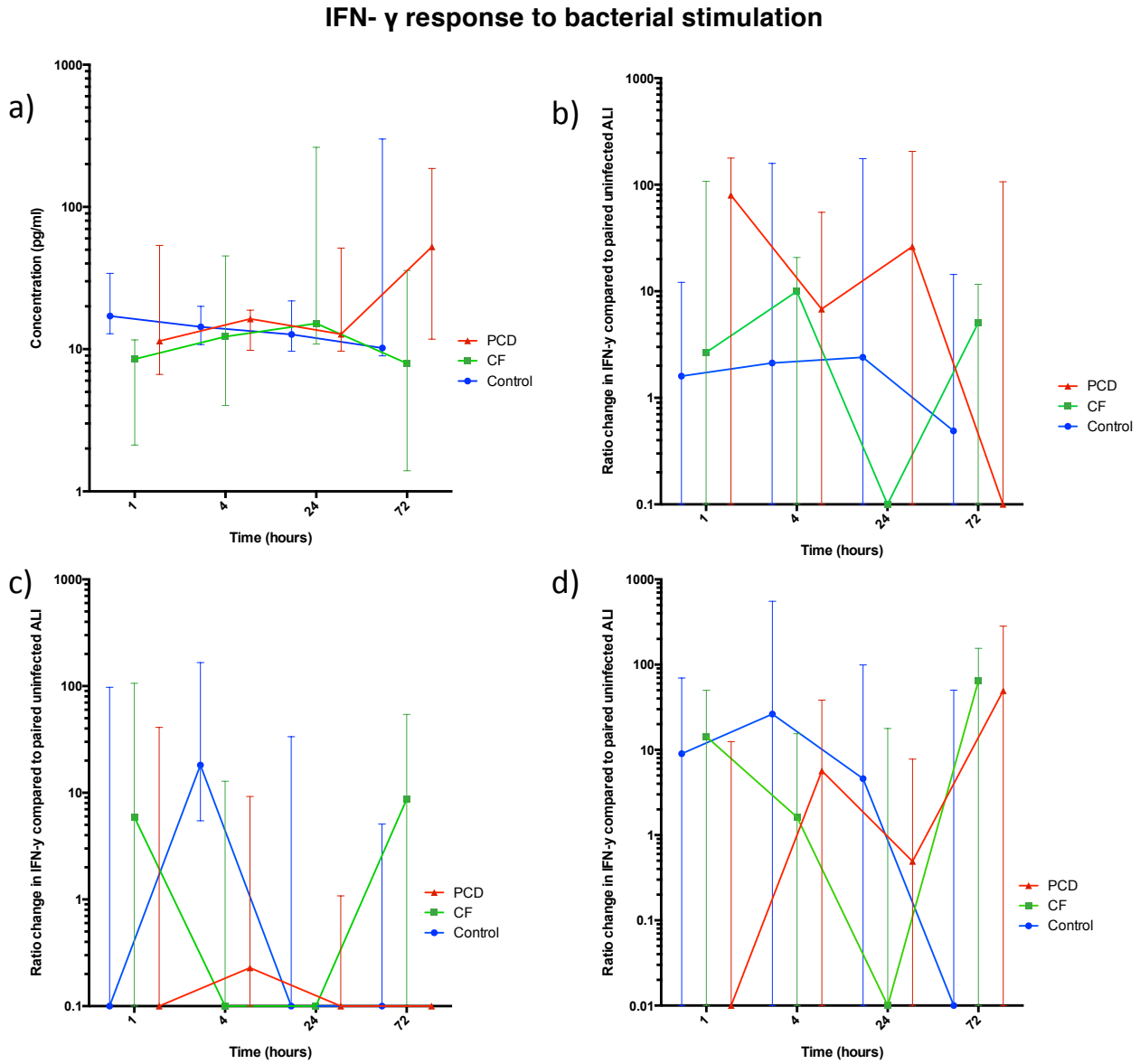


Figure 5.4-4: Levels of IFN- γ (pg/ml)

Figure 5.4-4: Levels of IFN- γ (pg/ml) shows no consistent rise in IFN- γ was detected following exposure to any of the pathogens.

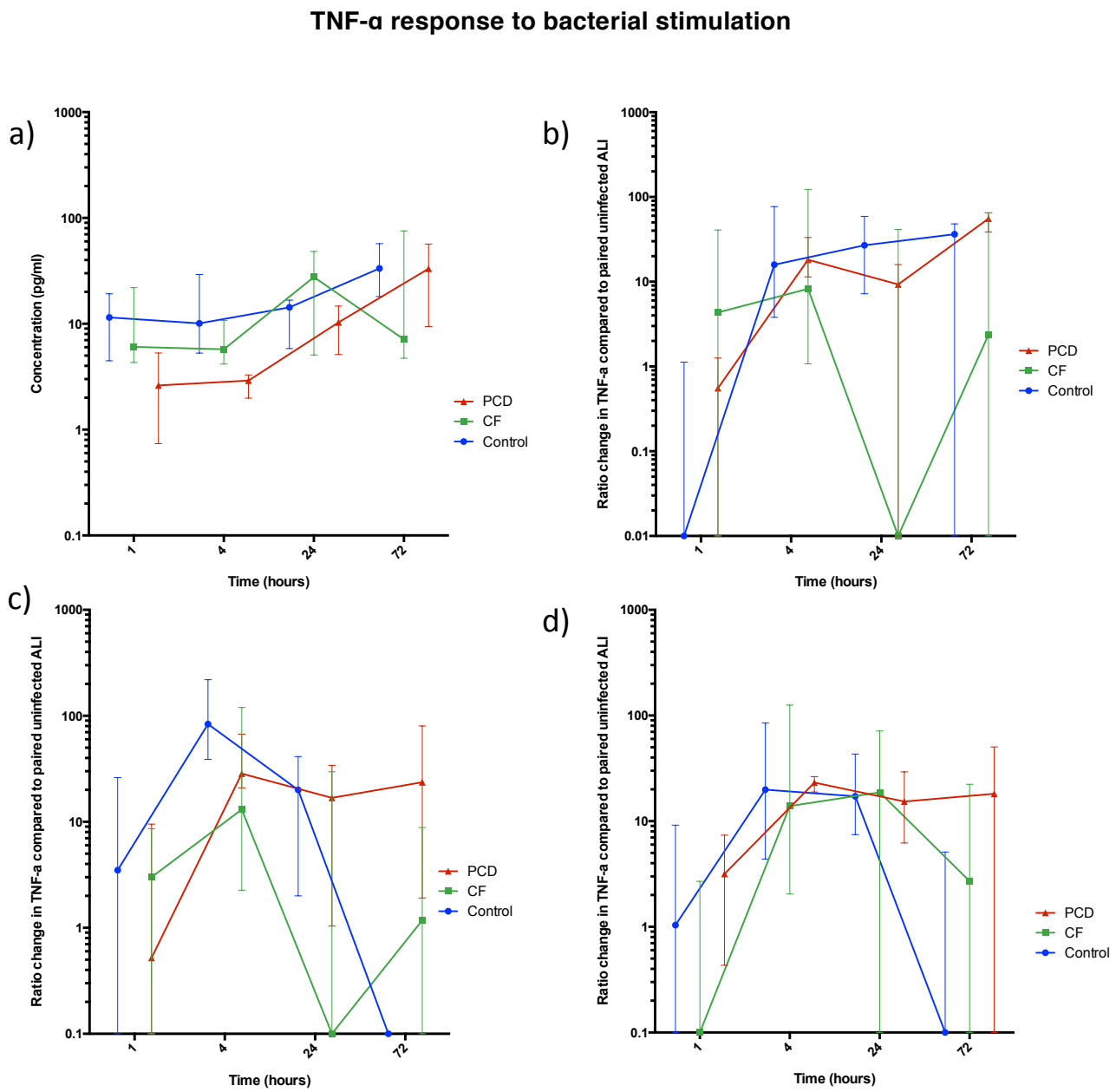


Figure 5.4-5: Levels of TNF-α (pg/ml)

Figure 5.4-5 demonstrates that there is a considerable rise in levels of TNF-α at 4 hours in all patient groups following exposure to all pathogens to a comparable extent, though this level did not increase from the 24 hour time point onwards. No significant difference was detected at 4 hours between TNF-α levels between the 3 patient groups.

The other 5 cytokines measured using the 9-plate pro-inflammatory cytokine kit did not display a significant response to bacterial exposure in any patient group.

5.4.2.3 Comparison of IL-8 levels measured on the Human ELISA Kit (Life Technologies) with the MSD) platform

The cell suspension samples that had been recorded to have ‘low’ and 'high' levels of IL-8 when analysed with the Life Technologies Human ELISA kits, also measured 'low' and 'high' levels respectively when analysed by the MSD platform (Table 5.4-1) indicating that the experiments could be interpreted along side each other.

IL-8 level (Life technologies ELISA)	MSD plate concentration (pg/ml)
Low	42.9
Low	44.2
High	15675
High	2986

Table 5.4-1: Comparison of IL-8 measurements with the Life Technologies ELISA Kit utilised in the cell suspension experiment and with the MSD platform

5.5 Discussion

The hypothesis stated that the cells from subjects with CF, with reduced/ absent CFTR function, would produce higher levels of pro-inflammatory cytokines than the other groups. The aim of this study was to define the relationship between CFTR function and inflammation by comparing the inflammatory response to bacterial challenge of CF nasal epithelial cells cultured at an ALI to those of wild type and non CF CSLD. This is the first study to simultaneously assess the inflammatory profile of epithelial cells obtained from subjects with CF, with those from patients with PCD and healthy controls. The level of inflammatory cytokines produced by epithelial cells from all 3 groups was measured at baseline, showing no difference between the groups, and then in response to bacterial stimulation by common respiratory pathogens over several time points. Cells from all groups showed elevated IL-6 levels, and a rise in TNF-α at 4 hours in response to bacterial stimulation, however there was no difference in the levels of elevated cytokines between the groups. An inflammatory response was not detected for other cytokines.

The pilot study revealed unexpected results, with CF and control primary cells in suspension demonstrated a three and five fold increase respectively in levels of IL-8 following exposure to respiratory pathogens compared to uninfected cells from the same subjects at 24 hours, whilst

PCD cells mounted a blunted inflammatory response. In the subsequent experiments undertaken by myself, the epithelial cells were cultured at an ALI, to better replicate *in vivo* conditions, prior to bacterial exposure. There was no significant difference in the levels of any of the inflammatory cytokines between PCD (4), CF (4) and healthy controls (5) at baseline. Some of the agar plates grew *SA* after the 24 hour collection of the supernatant, indicating that bacteria had not been successfully eradicated. This may have affected the inflammatory response due to unknown levels of continued bacterial stimulation by *SA,* however the cytokine responses were comparable to that seen with the other bacteria, so have been included in the analysis. There was an increase in the inflammatory cytokines IL-6 and TNF-α following pathogen exposure in all patient groups, illustrating an inflammatory response to the infectious stimuli. The levels of TNF-α peaked at 4 hours, whilst the IL-6 response was longer and consistently elevated for all patient groups, exposed to all 3 bacteria for up to 24 hours. There was no difference in the levels of either IL-6 or TNF-α at any of the time points between the 3 patient groups. A response was not detected for the other pro-inflammatory cytokines, which was a surprise, particularly for IL-8 given that a response had been detected in the pilot study.

There are several reasons that may explain the main study not replicating the positive findings of the pilot; both sample sizes were small, and so perhaps under-powered to show a true effect. Additionally different analysis platforms were used in the two experiments, and although the MSD platform provides improved sensitivity and dynamic range, it is possible that these kits were not comparable. Lastly, the cell populations used in the pilot study were not retested in the main study, so it is possible that the results reflect two cell lineages that could not be compared.

In the main study, the lack of inflammatory response across the range of cytokines could reflect either: no cytokine response elicited at this level of bacterial exposure, or the response was undetectable within the limits of the MSD platform and experimental methods. In order to establish the cause, further experiments refining the concentrations and conditions are required. A comparison of the IL-8 response between CF and PCD subjects would be particularly interesting as previous studies have reported a higher level of IL-8 in sputum from subjects with PCD, compared to subjects with CF [327].

Theses results support the previous studies that demonstrated no systematic differences in inflammatory cytokine production between CF and non-CF cells both at baseline or following pro-inflammatory stimulation (refs). The inclusion of subjects with PCD, who were shown to mount comparable levels of IL-6 and TNF- α to both other patient groups, suggests that defective MCC, common to PCD and CF, does not contribute towards a modified immune response. The level of

TNF- α was shown to peak earlier than IL-6, which may be as a result of the inhibitory effect of IL-6 on TNF-α, amongst other cytokines.

A large variability in the inflammatory response between individuals was observed for all patient groups. This is similar to other studies that have used human airway epithelial cell models, highlighting that genetic and environmental factors specific to the patients that provide the tissue samples contribute in a variable measure to the inflammatory response [317]. This wide degree of inter-cell variability makes it difficult to interpret subtle trends as there is a low signal-to-noise ratio. To mitigate against this effect, the experiment was designed to stabilise the conditions of each cell line, and care was taken to culture as many cell lines as possible, as discussed below.

1. Recent exposure to either bacterial or viral infections, or therapies may affect the response via altered inflammatory gene expression. The cells used in this experiment were cultured for a minimum of 6 weeks to help minimise these effects. In addition, the cells were taken back to a basal cell phenotype, re-differentiated and passaged twice so very few cells originally from the patient remained. However, larger numbers of subjects in each group would help to establish true trends, and determine if a difference in response, or variability exists between patient groups.

2. Despite a large number of subjects being recruited into the study (51), only 13 (25.5%) of the cilia brushing samples taken were successfully cultured to produce 4 wells of ciliated primary epithelial cell cultures. This percentage success rate was less than that for cells that are cultivated for clinical and research purposes by the PCD diagnostic service department, RBH; which was 32% when audited in 2013 and 52% in 2014. Prior studies have demonstrated lower rates of culture success in CF epithelial cells compared to non CF cells; thought to be as a results of the presence of opportunistic pathogens, CFTR mutation phenotype, and elevated airway IL-8 [329]. In addition, the audit included samples that were obtained when the patient was under anaesthetic for a bronchoscopy, which yield a better cell count.

These results showed that whilst suspended nasal epithelial cells from patients with PCD have an attenuated inflammatory response, this is not intrinsic to the PCD cellular phenotype, as demonstrated by a rise in cytokines following bacterial stimulation when cultured at ALI. The pilot study may however accurately reflect the *in vivo* behaviour, with the blunted response demonstrated in PCD cells a result of the effects of chronic nasal infection (references) producing altered inflammatory gene expression and also, as hypothesized in this thesis, secondary CFTR dysfunction. Chronic upper airway infection is more common in PCD than CF and controls,

accounting for the difference in response between the patient groups. This study highlights that *in vitro* model systems for studying airway epithelial cell behaviour may not replicate the complexity of the airway. In particular, interpretation of the ALI results comes with the caveat that it illustrates only the response of nasal epithelial cells cultured in a controlled environment, and may not exactly replicate bronchial epithelial cell reactions *in vivo.*

This model does not support the hypothesis that cells with reduced CFTR function (CF), at an ALI, give rise to excessive inflammatory cytokines, it does not explore the effect of dysfunctional CFTR in many other elements of the immune system, as discussed Chapter 1, namely, myeloid-derived cells, neutrophils, macrophages [96, 330-333]. It is possible that a combination of many of these pathways affect the immune response in patients with CF and contribute, at different stages, to the chronic inflammatory picture characteristic of the disease. A better understanding of the exact mechanisms may provide future potential approaches for dampening inflammation by targeting specific cells or interactions.

Study limitations and future work

This study has several limitations. Crucially, the group numbers were very small meaning that the study was underpowered. Ideally this experiment would be continued to increase patient numbers to account for the large variability in data. A further potential weakness of this study is that different multiplex ELISA platforms were used in the cell suspension protocol and pilot studies; the Life Technologies Human ELISA compared to the MSD plate used to analyse the cell culture supernatant in the main study. IL-8 levels in cell suspension samples measured with both platforms revealed results of a similar magnitude suggesting that the 2 sets of data could be interpreted along side each other, however experimental conditions need to be refined in order to compare cytokines like for like. It is essential for future work to determine the dose-cytokine response relationship for each bacterium, which lies within the dynamic range of the MSD plate, and compare the top dose of each in order to draw conclusions for different bacterial stimulation. Following this, repeat measurements on cell cultures from the same donor, and from the same supernatant samples are required to ensure reliability of the measurements.

Furthermore, this study makes the assumption that CFTR function in the cells of patients with PCD is normal and any dysfunction is limited to the CF subjects. There is emerging evidence that CFTR function might be affected by localised inflammatory mediators, which is explored further *in vivo* in the next chapter. Culturing PCD cells *ex-vivo*, meant that secondary CFTR function was unlikely, however future experiments should quantify the CFTR function using a patch clamp.

Summary

In summary, cells with absent CFTR function, from subjects with CF, when cultured at an ALI, produced a similar level of inflammatory mediators at baseline and following bacterial exposure to cells from patients with PCD and healthy controls. This does not support the hypothesis, that absent CFTR function results in hyperinflammatory epithelial response.

However, these *in vitro* experiments may not mimic *in vivo* cellular and molecular interactions, so in the next chapter I have studied the interactions between CFTR function as demonstrated by nPD and airway inflammation *in vivo*.

6 The relationship between secondary CFTR dysfunction and inflammation in subjects with Primary Ciliary Dyskinesia

6.1 Introduction

Impaired CFTR function in CF is caused by a genetic defect in the CFTR gene, resulting in abnormal expression, or structure and function of the protein. There is increasing evidence to suggest that CFTR ion channel function can also be affected by external modulators, resulting in a secondary dysfunction. Section 1.2.4 provided a detailed overview of the studies that have been performed to explore the effect of inflammatory mediators and infective microbes on CFTR function, followed by the evidence of CFTR dysfunction in other respiratory diseases, which to date has focused on COPD. Together, these results indicate that external factors may cause an acquired dysfunction in CFTR. These findings have implications for subjects with CF, and for other respiratory conditions, in which the presence of abnormal ion transport may contribute to the burden of disease.

To date, no studies have measured CFTR function in patients with PCD, which has a similar pathophysiology to CF, with defective MCC, resulting in a comparable inflammatory profile. This chapter describes a study designed to explore the relationship between localised inflammation and CFTR ion channel function, measured in vivo in subjects with CF, healthy controls and patients with PCD, to determine whether there is evidence of secondary CFTR dysfunction in PCD.

This study aims to test part of my second hypothesis, namely that dysregulated chloride and sodium transport leads to inflammation and inflammation which in turn further impairs ion transport, which will be tested in another chronic suppurative lung disease, PCD.

6.2 Study aim:

- To define the relationship between CFTR function as demonstrated by chloride and sodium transport, and inflammation by measuring NPD in patients with PCD, and relating it to the severity of nasal inflammation

Objectives;

- To perform NPD measurements and compare the indices; basal values, amiloride response and chloride secretion, in patients with CF with reduced/absent CFTR function (positive controls), patients with PCD and healthy (negative) controls
- To measure the levels of inflammatory cytokines in nasal secretions by nasosorption (upper airway), and to correlate these markers of inflammation with levels of CFTR function

The protocol was written by Dr Suzanne Crowley and Professor Jane Davies. All subject recruitment, data collection and analysis and statistics were performed by myself.

6.2.1 Recruitment and consent

Recruitment and study visits took place between 1 April 2013 and 30 September 2014. The diagnosis of PCD, CF and healthy controls were confirmed as described in Section 2.1. Patients with PCD were recruited from outpatient clinics at the RBH. Patients were identified as eligible by advance screening of electronic clinic lists, followed by a discussion with their lead clinician to ensure suitability. Patients were asked to attend for a study visit at a time of clinical stability. Patients with CF were recruited from in-patients in the ward. Eligibility was confirmed by reviewing electronic notes followed by a discussion with the clinical team. Patients were approached whilst on the ward and the trial visit was performed at the end of their stay, when their lead clinician deemed them to be clinically stable. Healthy controls were recruited as described in Section 2.1.

Subjects were given the PIS 1 week before the study visit, and given the opportunity to ask questions and discuss to study, prior to obtaining consent.

6.2.1.1 Inclusion and exclusion criteria

Inclusion criteria

Males and females aged 16 years or above

A stable baseline state of health and free of viral infection in the preceding 4 weeks.

Non-smokers, based on patient history

Exclusion criteria

Infection with Burkholderia cepacia complex or MRSA (for infection control reasons)

Significant nasal pathology including polyps, clinically-significant acute rhinosinusitis, or recurrent severe epistaxis

Acute upper respiratory tract infection within the last 4 weeks

6.2.2 Ethics

Prior approval was given by the NRES Committee London-Brent (REC: 13/LO/0391)

6.2.3 Study design

All measurements were performed on a single day at the Clinical Research Facility, RBH, London. Subjects consented to have the following investigations performed:

6.2.3.1 Investigations

The following investigations were performed on all subjects:

- Baseline height and weight measurements
- CFTR mutation analysis (unless result already available)
- Complete a short medical questionnaire
- Spirometry
- Sweat test (unless result already available)
- Nasal potential difference (NPD)
- Nasosorption

CFTR mutation analysis was performed on all subjects if the results were not already available. This was used to ensure that no patient in either the PCD or control group carried a single CFTR mutation, which may affect the results. Spirometry was performed as a generalised marker of airway disease and measurements of localised inflammation were obtained by performing nasosorption. NPD measurements were used as surrogate markers of CFTR function in the nasal

epithelium. Sweat chloride measurements were performed to assist in the interpretation of results and determine whether any potential secondary CFTR dysfunction is localised or systemic.

Nasosorption, using synthetic absorptive matrices (SAM) was chosen to sample nasal mucosal lining fluid (MLF). This method has the advantage that it is non-invasive, and it is possible to sample neat MLF, allowing detection of higher levels of chemokines and cytokines than after nasal lavage. It has recently been employed in children with rhinitis and in relation to nasal allergen challenge [334, 335]. The samples were analysed on a multiplex immunoasssay designed to simultaneously quantitate the presence of multiple cytokines including IL-1β, IL-6, IL-8, IL-10, IFN-γ and TNF-α. This plate was chosen as these cytokines have all been previously measured in nasal mucosal lining fluid from nasosorption samples, and demonstrated a dose dependent response to a lipopolysaccharide challenge which is a stimulus to the innate immune system [239].

6.2.3.2 Sample size

Previous studies have derived the sample sizes needed to detect a within group differences in total chloride response using NPD measurements, demonstrating that a patient group between 30 and 40 provides a 90% power to demonstrate a difference in chloride response of -5mV [296].

6.3 Method

25 subjects consented to participate: PCD n=8, CF n= 9, and healthy control n=9. 3 subjects were subsequently excluded as it was revealed that 1 subject (PCD) had received laser surgery on his nostril, 1 subject was unable to tolerate NPD measurements (CF) and 1 subject was not well on the day of the appointment and was unable to re-attend (control). This is less than the intended number of subjects due to both recruitment difficulties and time limitations; hence the study is underpowered, however it provides useful pilot data to take forward to a larger study.

All measurements were performed on the same day. The methods used to measure height and weight, to perform spirometry and for CFTR mutation detection are described in Sections 2.2.1, 2.2.2, and 2.2.7.

Care was taken to insure that nasosorption was performed prior to the NPD measurements, as it was considered that the perfusion of solutions on the nasal mucosa might affect the nasosorption results. Other interventions were performed in any order depending on the machine and equipment availability.

6.3.1 Medical questionnaire

All subjects were asked to fill in a short medical questionnaire to obtain information about their previous medical issues and current status (Table 6.3-1)

Chest problems	Yes	No	Comments
Neonatal chest problems			
Current chest problems			
Age of onset and details:			
Persistent cough			
Purulent sputum			
Family history			

Nose and ear problems	Yes	No	Comments
Neonatal nose problems			
Current nose problems			
Age of onset and details:			
Pooled pus in nose			
Purulent sputum			
Sinus involvement			
Any sinus surgery			

Table 6.3-1: Symptom questionnaire of past and current chest an ENT symptoms

6.3.2 Sweat test

Sweat test was performed as described in Section 2.2.6. It was not repeated if it had previously been performed for clinical reasons and the information was available. The value taken forward for the analysis was the mean of the chloride concentration (mmol/L) for both arms.

6.3.3 Nasal Potential Difference (NPD)

NPD measurements were performed and the individual indices calculated as described in Section 2.2.8.1.

Analysis of the NPD traces

2 methods of scoring NPD measurements, devised by Wilschanski and by Sermet, have been developed to attempt to quantify CFTR function in nasal epithelium. These analyses take into account measurements of both the CFTR-dependent sodium and chloride ion transport, and provide a globalised measure of ion transport across the entire NPD trace [336] [337].

Wilschanski index: values are calculated using the equation $e^{\Delta \text{ chloride}/\Delta \text{ amiloride}}$. A cut off value of >0.70 has been shown to predict a diagnosis of CF, with values greater than 0.7 consistent with CF.

Sermet: values are calculated using the equation -0.11 Δ low Cl-/Iso – 0.05 Δ amiloride. A diagnostic cut off of <0.27 was shown to discriminate patients with CF (values <0.27) from healthy controls.

The Wilschanski index was shown to better differentiate between atypical CF and non-CF patients than analysis of either sodium or chloride components alone. The method devised by Sermet was shown to be useful diagnostic tool for young children with inconclusive investigations at newborn screening, and further validated in an adult population of patients with CF and controls.

For all traces performed in this study, both the Wilchanski index and the Sermet score were calculated to see if the these methods demonstrated a difference in CFTR function between patients with PCD and healthy controls, and how this compared to patients with CF.

6.3.4 Nasosorption

Nasosorption was performed using SAM strips, which are comprised of synthetic fibrous hydroxylated polyester medium (Accuwick Ultra, Pall) cut into two strips, designed to absorb nasal secretions for analysis of the levels of cytokines and chemokines.

6.3.4.1 Collection:

With the patient sitting comfortable upright and head extended backwards, a SAM strip was placed under direct vision, on the lateral wall of the nasal cavity, using blunt ended forceps if required. Light pressure with the patient's finger was used to ensure that the SAM remained in place for 1 minute. The process was repeated, with fresh strip, on the other nostril. Strips were immediately placed in a Corning Costar Spin-X centrifuge tube filter, with 500 μl of chilled 0.9% saline and centrifuged at 16,000g for 10 minutes, cooled to 4^{o}C. The filter cup with the dry SAM was removed and discarded, and fluid retained in the tube was stored in Eppendorfs at -80^{o}C.

6.3.4.2 Sample analysis:

The samples were thawed and the cytokine profile measured using the MSD platform for the presence of 9 proinflammatory cytokines: GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, and TNF-α as described in Section 2.2.5.2.

To assess the repeatability of the nasosorption values, 2 aliquots were taken from the same Eppendorf tube from samples from 6 of the patients with PCD, and analysed on the same MSD plate. No further samples were analysed in duplicate due to space limitation on the MSD plate.

6.3.5 Statistics

Data were analysed using Prism 6 statistical software (GraphPad Software Inc, CA, USA). Data are presented as median and interquartile range (IQR). Non-parametric paired comparison of pre treatment and post treatment values were analysed using the Wilcoxon matched-pairs Signed Rank Test. Correlation between two outcome measures were assessed with Spearman's rank correlation coefficient and p values < 0.05 were considered significant.

6.4 Results

The demographic data are summarised in Table 6.4-1. Subjects were comparable for age and gender.

	Control	CF	PCD
Total no:	8	8	7
Gender: female (%)	63	50	57
Age: median (IQR)	31.1 (25.0-34.9)	27.4 (24.9-28.93)	35.6 (28.3-43.4)
CFTR Genotype	None detected	All F508del/F508del	None detected
PCD Defect	N/A	N/A	4 ODA 1 IDA+ODA 1 Transposition 1 Normal (HYDIN gene)

Table 6.4-1: Baseline characteristics of the subjects. The total numbers of patients recruited into each group are listed along with the age (years) median (IQR), gender, CF genotype or PCD defect/ gene. Outer dynein arm defect = ODA, inner dynein arm defect=IDA.

6.4.1 Questionnaire

The results of the questionnaire are summarised in

Table 6.4-2. All of the subjects with CF reported chest symptoms as many of them were at the end of an admission for a pulmonary exacerbation. More subjects in the PCD group reported ongoing nasal symptoms than the CF group and controls, in keeping with the known distribution of the disease.

Group	Time	Nasal symptoms	Sinus involvement	Chest symptoms
Control (n=8)	Current	0	0	0
	Past	0	0	0
CF (n=8)	Current	0	0	0
	Past	1	2	8
PCD (n=7)	Current	0	0	1
	Past	4	3	5

Table 6.4-2: Results from the symptom questionnaire. The number of subjects from each subject group who reported symptoms as either ‘current’ or ‘past’ is documented.

6.4.2 Spirometry

Acceptable measurements were achieved in all 23 subjects. The FEV_1 (% predicted) was significantly lower in the CF group, with a median (IQR) of 50.1 (38.1-58.6) compared to the healthy controls, median (IQR) 88.7 (82.4-101.1) % predicted ($p<0.001$). The values for subjects with PCD lay between these 2 groups with a median (IQR) of 69.8 (64.4-85.3) % predicted (not significantly different to either group). Similar results were seen when comparing values for FVC (% predicted).

6.4.3 Sweat Chloride

Results for sweat chloride concentration were obtained in only 22/23 subjects as 1 sample was an insufficient quantity for analysis (healthy control). The results were as expected for the patient groups; with CF subjects demonstrating a significantly higher concentration of chloride in the sweat sample with a median (IQR) mmol/L of 99.5 (98.3-106.3) ($p<0.05$) than patients with PCD (18.5 (14.0-29.5) mmol/L) and healthy controls; 19.5 (12.5-37.5) mmol/L).

6.4.4 Nasal PD

The traces for all patients (23) were reviewed and considered to be of acceptable quality. For 1 subject with CF, the trace was of poor quality after the perfusion of ZCl solution and for this trace only the basal values and amiloride phase are used in the analysis. The basal values are presented in Figure 6.4-1, illustrating that patients with CF show a significantly more negative reading median (IQR) mV: -52 (-51 - -60) compared to PCD, -17 (-16 - -24) mV, and control, -24 (-19 - -28) mV.

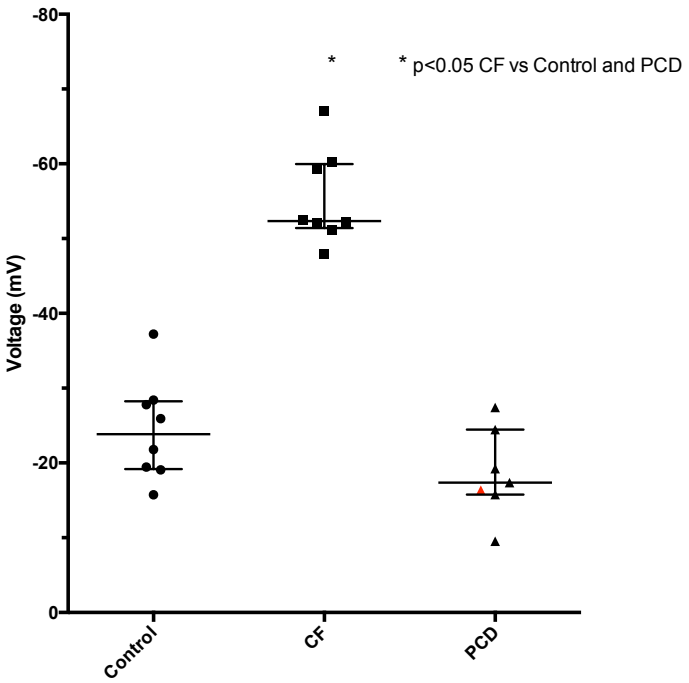


Figure 6.4-1: Comparison of baseline voltage (mV) between study groups demonstrating that subjects with CF have a significantly more negative basal PD than both control and PCD subjectsc. *Each patient is represented by a single data point with the median (mV) and IQR indicated*.

The changes in voltage following perfusion of amiloride solution are displayed on Figure 6.4-2 illustrating the absolute change, and change expressed as a percentage of the baseline respectively. The greatest change was seen in the CF patients, with a median (IQR) change of +34.3 mV (25.4 – 38.2), reflecting the over-activity of ENaC, which relates to absence of CFTR function. Controls showed a moderate change; median (IQR) +16.61 mV (8.8 -19.7), consistent with previous data whilst the patients with PCD showed only a very minimal change with a median (IQR) of +3.6 mV (0.0-10.2) [338]. There was a statistical difference between the amiloride response between the PCD subjects and the CF group, though not between the PCD and the control group. Furthermore there was a statistical difference between the percentage of amiloride response compared to the baseline between PCD and CF (with PCD showing a reduced response), though not between PCD and control. These results suggest no disinhibition of ENaC activity in the PCD group, and contrary to expected either normal or reduced ENaC function.

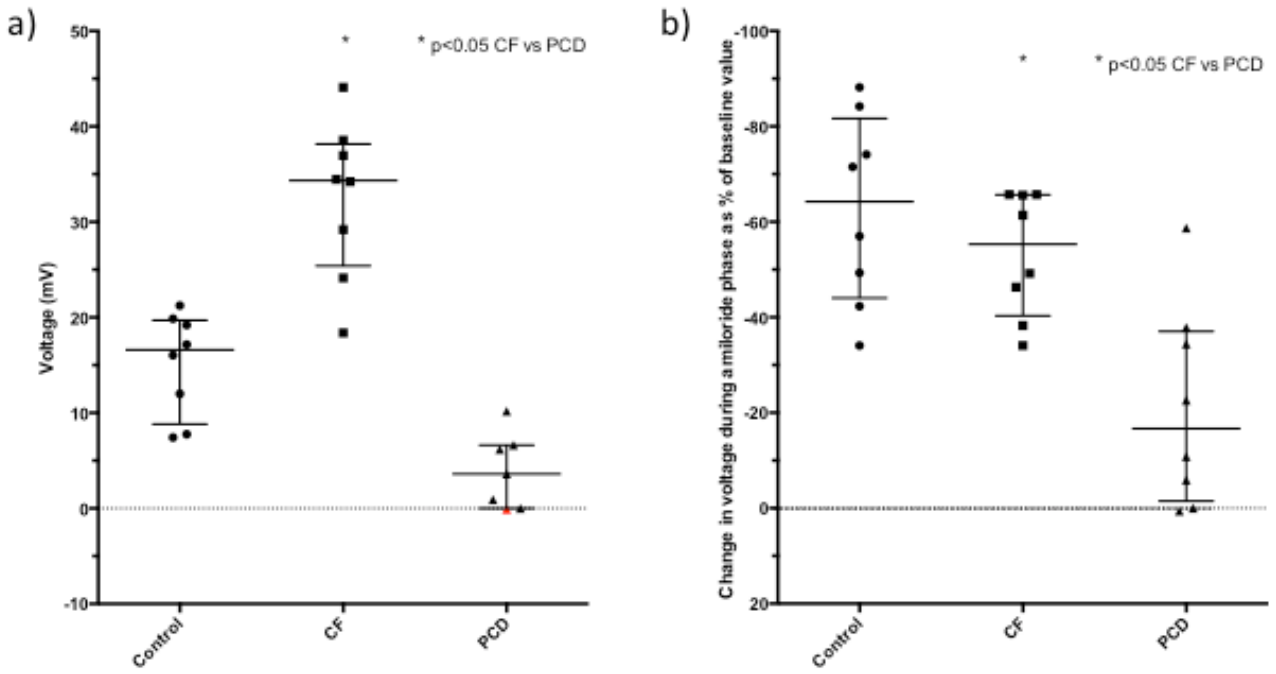


Figure 6.4-2 a) Absolute change in voltage (mV) following perfusion of Ringers/Amiloride and b) amiloride response expressed as a percentage (%) of the baseline demonstrating a difference between CF and PCD, however not between either of these groups and controls. *Each patient is represented by a single data point with the median (mV) and IQR indicated.*

Figure 6.4-3 shows the total change in voltage following the perfusion of both the ZCl and ZCl/Iso solutions (reflecting the total chloride secretion). The control group showed a significantly larger change in voltage with median (IQR) -35 mV (-24 - -54), compared to the CF group -0.97mV (+3.4 - -3.7). The PCD group showed significantly reduced chloride secretion compared to controls, with a median of -7 mV (-3.6 - -17), which was comparable to that seen with subjects with CF ($p<0.001$).

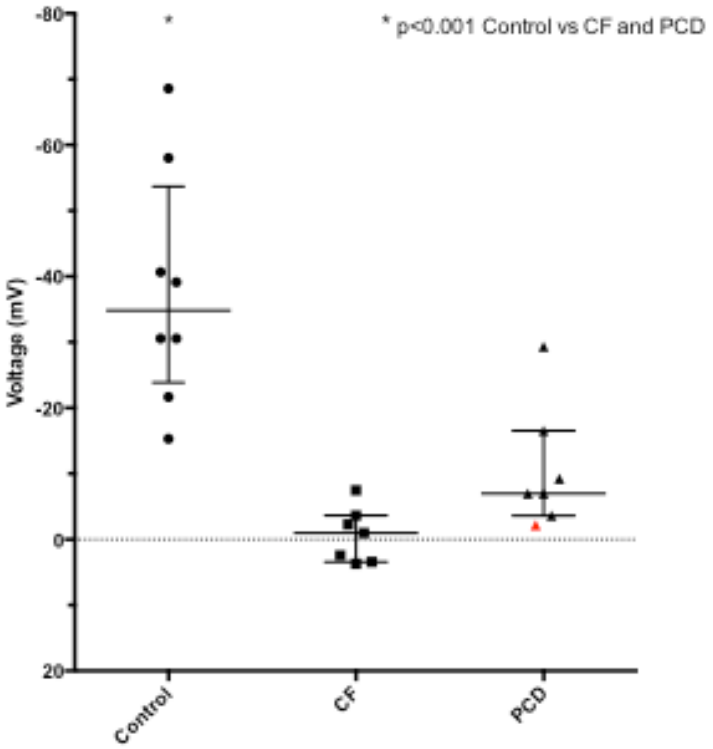


Figure 6.4-3: Comparison of total chloride secretion (mV) between study groups showing a significant difference between the control group and both the CF and PCD group. *Each patient is represented by a single data point with the median (mV) and IQR indicated.*

The Wilchanski index and Sermet score were calculated for the individual traces to take account of both the sodium and chloride transport elements of the NPD, illustrated in Figure 6.4-4. It was not possible to calculate the Wilchanski index for one of the subjects with PCD, as the amiloride phase had resulted in a small negative deflection. The Sermet score was calculated for all traces.

The Wilchanski index indicated a difference between PCD and controls in 4 of the 6 traces analysed and the Sermet score in 2 of the 7.

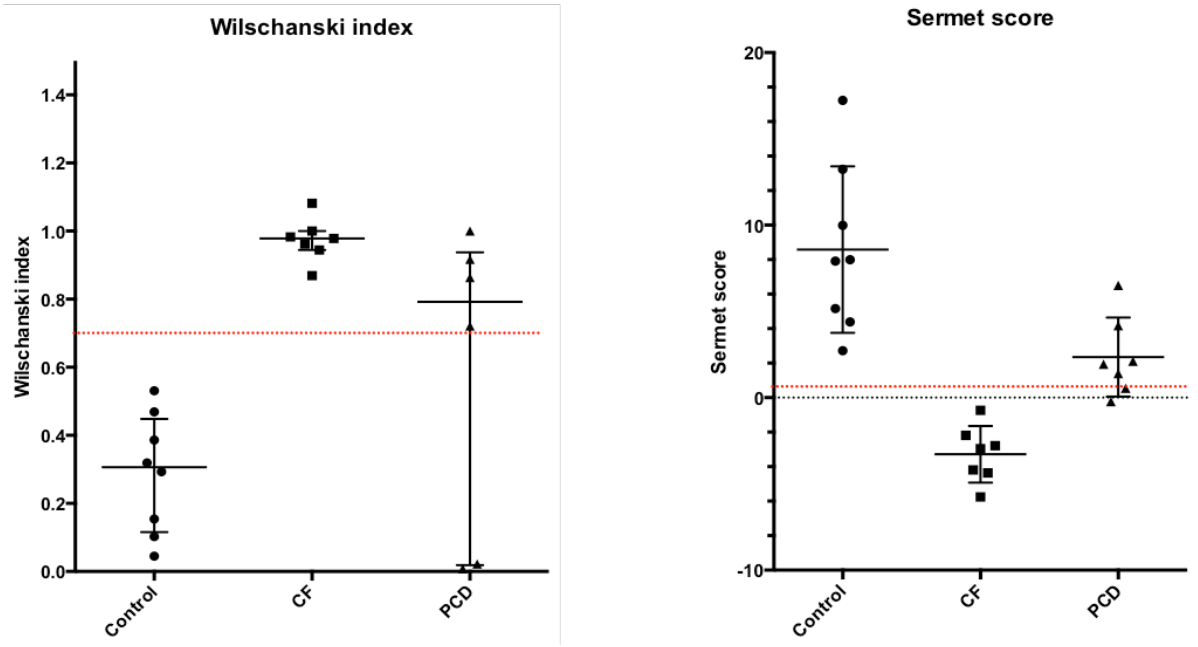


Figure 6.4-4: Evaluation of CFTR function using global scoring systems a) Wilchanski index where the dotted line represents the cut off above which indicates abnormal CFTR function and b) Sermet score where the dotted line represents the cut off below which indicates abnormal CFTR function. *For each patient the score was calculated and represented as a single data point with the median an IQR range indicated.*

Of the 6 PCD traces in which both scoring systems could be performed; 2 were categorized as normal CFTR function with both indices, 2 had abnormal CFTR function compared to healthy controls using the Wilchanski index alone nil with Sermet score alone, and 2 by both illustrated in Figure 6.4-5)

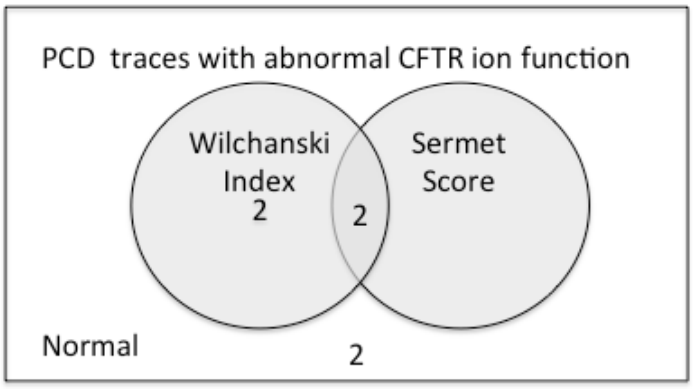


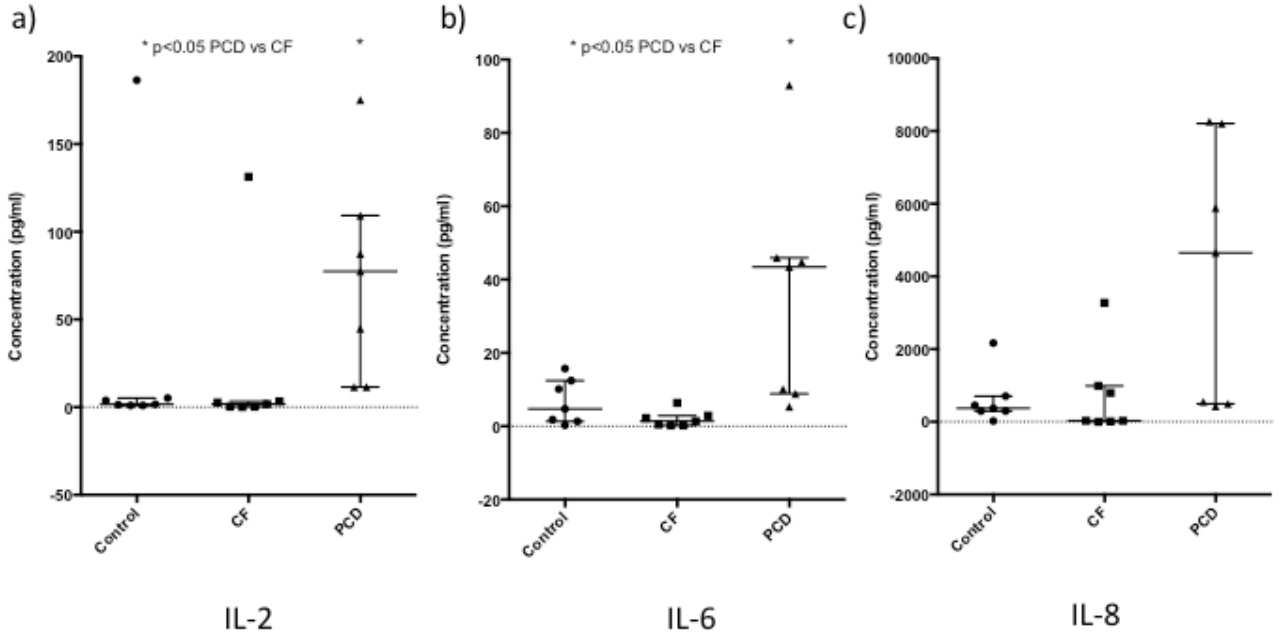
Figure 6.4-5: Venn diagram demonstrating how the NPD traces from subjects with PCD were categorised according to Wilchanski index and Sermet score. The traces in which the CFTR function was categorised as abnormal are documented.

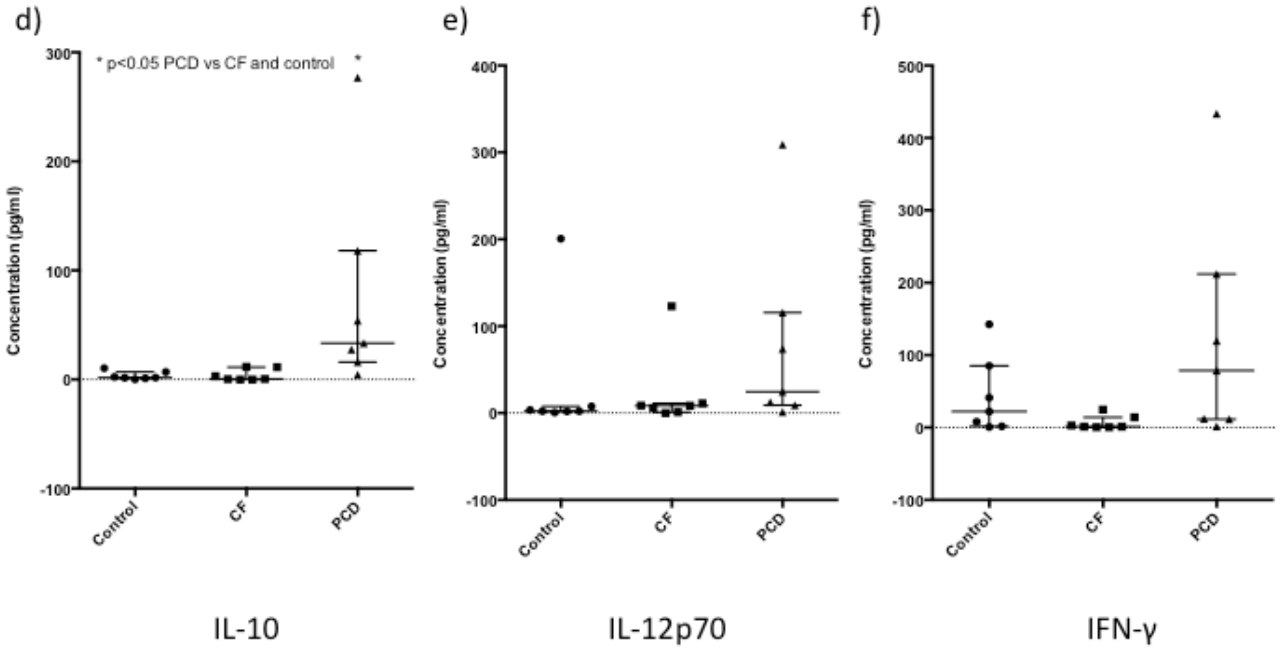
6.4.5 Nasosorption

Nasosorption samples were collected on 22/23 subjects. Results are unavailable on 1 control subject due to equipment failure, and only a single nostril was sampled on 1 patient with CF as the subject found the procedure uncomfortable. In total 41 samples were analysed: control 14 (2x7), CF 13 (2x6 +1) and PCD 14 (2x7). 2 aliquots from the same samples were analysed on the MSD plate for 6 of the patients with PCD from both the right and left nostril samples (12 samples in total analysed in duplicate). This was only performed in the PCD group due to space limitation on the MSD plate.

6.4.5.1 Comparison of patient groups:

The results for the nasosorption samples for each cytokine are displayed below in Figure 6.4-6.The graphs illustrate a trend towards increased levels of all cytokines in nasal secretions in subjects with PCD compared to the CF and healthy control groups, which reached statistical significance for PCD vs CF for: IL- 1β, IL-2, IL-6, IL-10, TNF-α, IFN-γ, and PCD vs healthy control: IL-10. In particular, it was 3 of the subjects in the PCD group that showed consistently high levels across the range of cytokines


a)
* p<0.05 PCD vs CF
Concentration (pg/ml)
Control
CF
PCD
IL-2
b)
* p<0.05 PCD vs CF
Concentration (pg/ml)
Control
CF
PCD
IL-6
c)
Concentration (pg/ml)
Control
CF
PCD
IL-8


d)
* p<0.05 PCD vs CF and control
Concentration (pg/ml)
Control
CF
PCD
IL-10
e)
Concentration (pg/ml)
Control
CF
PCD
IL-12p70
f)
Concentration (pg/ml)
Control
CF
PCD
IFN-γ

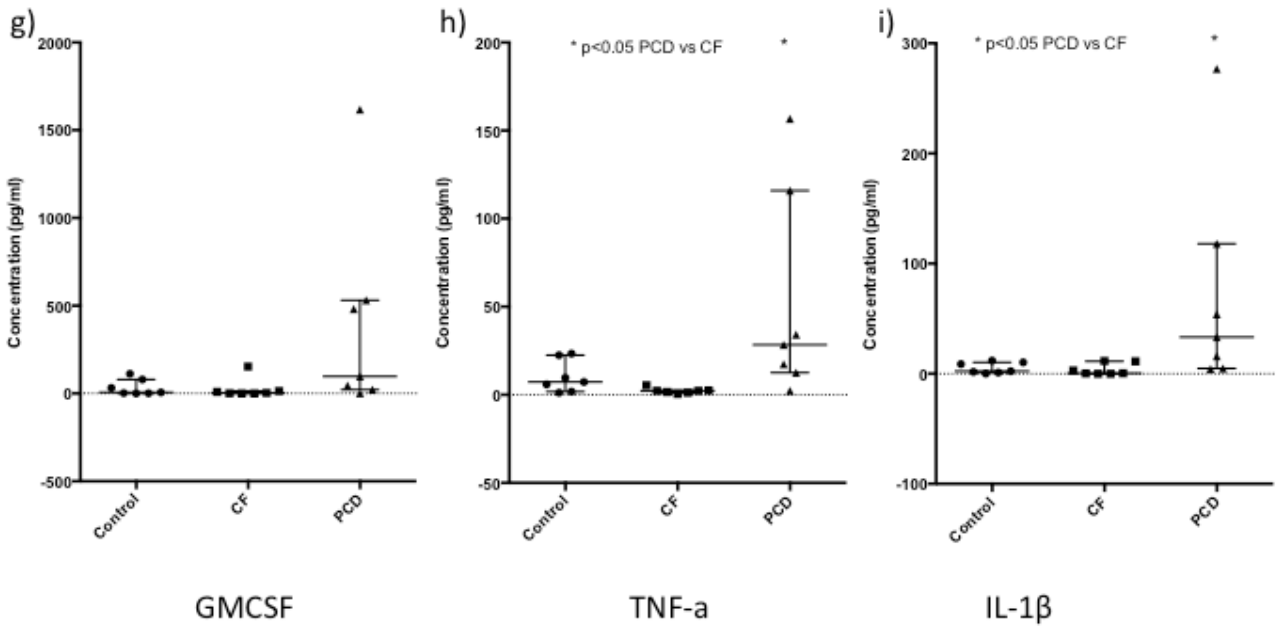


Figure 6.4-6: Cytokine measurements for each subject group. a) IL-2 b) IL-6 c) IL-8 d) IL-10 e) IL-12p70 f) IFN-γ g) GMCSF h) TNF-α i) IL- 1β. Results in which there was a statistical difference between PCD and the other groups are indicated. *Each patient is represented by a single data point with the median (pg/ml) and IQR indicated.*

6.4.5.2 Spread of the cytokine levels

Table 6.4-3 lists the upper and lower limits of detection for each cytokine assay, along with the number of measurements for each group which were outside this range. Most values were near the lower limit, with more below the level of detection in controls and CF than PCD, and none above the level of detection.

Cytokine	Dynamic range	Control		CF		PCD	
		Below	Above	Below	Above	Below	Above
GM-CSF	0.29-10000 pg/mL	1	0	1	0	0	0
IFN-γ	6.17-10000 pg/mL	3	0	5	0	1	0
IL-1β	0.42-10000 pg/mL	1	0	4	0	0	0
IL-2	0.32-10000 pg/mL	0	0	3	0	0	0
IL-6	0.40-10000 pg/mL	1	0	2	0	0	0
IL-8	0.17-10000 pg/mL	0	0	1	0	0	0
IL-10	0.22-10000 pg/mL	1	0	2	0	0	0
IL-12p70	0.32-10000 pg/mL	0	0	1	0	0	0
TNF-α	0.90-10000 pg/mL	0	0	1	0	0	0

Table 6.4-3: Display of how many measurements fell outside the limits of detection of the MSD plate for each group

6.4.5.3 Variability of sample results:

To calculate the coefficient of variation (CV%), values that lay outside the limit of detection were excluded.

Intra-patient (between nostril) variability

The CV% for the 2 values obtained for each cytokine from each nostril was calculated for all of the subjects. These are illustrated in Table 6.4-4 as the median CV% (IQR) for each group for each cytokine. These demonstrate a large variation between samples obtained from different nostrils in all groups with no statistical difference between control, CF and PCD.

Cytokine	Co-efficient of variation (CV)%		
	Control	CF	PCD
GM-CSF	50.3	51.2	37.3
IFN-γ	95.5	58.1	34.2
IL-1β	79.9	35.3	71.5
IL-2	43.5	25.7	13.3
IL-6	69.2	53.6	40.6
IL-8	62	38.4	65.9
IL-10	71.8	35.3	67.4
IL-12p70	74.5	33.9	50.1
TNF-α	50.3	51.2	65.5

Table 6.4-4: Coefficient of variation (%) of the cytokine measurements in the nasosorption samples between each nostril for control subjects, CF and PCD. For each cytokine the CV (%) for the measurements of the 3 groups are illustrated. *Data are presented as median.*

Intra-sample variability

Following this, for the 6 patients in the PCD group, the CV% was calculated for the 12 samples (6 right nostril, 6 left nostril), which had been analysed in duplicate. These are illustrated in

Table 6.4-5. The results demonstrate extremely large variation in the results obtained from the same sample across all 9 cytokines.

Cytokine	CV (%)
GM-CSF	66.15
IFN-γ	59.81
IL-1β	36.72
IL-2	23.2
IL-6	48.03
IL-8	57.51
IL-10	49.78
IL-12p70	45.63
TNF-α	54.56

Table 6.4-5: Coefficient of variation (%) of nasosorption samples taken from the same sample from the same nostril in patients with PCD. Data are presented as median and IQR.

As the CV (%) was comparable between samples taken from the same aliquot and analysed in duplicate and the samples taken from different nostrils, the mean of cytokine concentration between both nostrils for each subject was taken forward for further analysis.

6.4.6 Relationship between the level of inflammatory cytokines and CFTR function in subjects with PCD

The levels of CFTR function in patients with PCD, as shown by sodium transport (basal PD and amiloride response) and chloride transport (total chloride response) were compared to the levels of inflammatory cytokines; IL-2, IL-6, IL-10, TNF α and IL-1β as these were the only ones significantly elevated. The results are tabulated in

Table 6.4-6, revealing no correlation between these parameters and levels of inflammation.

	IL-2		IL-6		IL-10		IL-1β		TNF-α	
	r	p	r	p	r	p	r	p	r	p
Basal (mV)	0.00	>0.99	-0.14	0.78	0.32	0.50	0.36	0.44	-0.21	0.66
Amiloride response	0.21	0.66	0.25	0.59	0.61	0.17	0.50	0.27	0.46	0.30
Chloride secretion	-0.29	0.56	-0.11	0.84	-0.36	0.44	-0.46	0.30	0.11	0.84

Table 6.4-6 Relationship between selected cytokines and parameters derived from NPD measuring CFTR ion channel transport function, revealing no correlation. *r and p values are tabulated, calculated using spearman's rank coefficient*

The inflammatory cytokines were elevated consistently in 3 of the PCD subjects. All of these 3 patients had reported past nasal or sinus symptoms in the questionnaire, in contrast to only 1 of the other 4 PCD patients.

6.5 Discussion

This is the first study to assess CFTR function *in vivo* in subjects with PCD. The aim was to define the relationship between the function of the CFTR protein as shown by chloride and sodium transport and the severity of local inflammation in this condition. CFTR function was evaluated using NPD and the relatively novel technique of nasosorption was used to assess inflammation in the nasal secretions. The NPD traces of patients with PCD suggested normal basal values, normal or reduced amiloride responses, and evidence of reduced chloride secretion. Patients with PCD

also had higher levels of inflammatory cytokines in nasal MLF than either the controls of subjects with CF, particularly in 3 of the subjects. Levels of inflammatory cytokine did not correlate with either CFTR function measured by sodium or chloride transport. These results are discussed below in turn.

The groups were comparable for age and gender, and as expected, the subjects with CF had significantly worse lung function than the controls, with values for the patients with PCD lying between these two groups. All the sweat chloride values were in keeping with those from previous studies for each group.

6.5.1 Nasal potential difference

The NPD measurements are interesting and can be interpreted in different ways. The NPD traces in subjects with CF with impaired CFTR ion transport function are characterised by a high (more negative) basal value, a large amiloride response and no change in voltage upon perfusion of chloride free solution, with the first two values reflecting sodium hyperabsorption (lack of inhibition of ENaC) and the final measurement demonstrating reduced chloride secretion. In this study, the patients with PCD showed reduced chloride secretion, comparable to that seen in the subjects with CF, however this was not accompanied by a correspondingly high basal value, and the amiloride response was surprisingly shown to be less than normal. Secondary analysis of the traces using the Wilchanski index and Sermet analysis; which combine the contribution of the sodium and chloride ion transport to evaluate evidence of CFTR function, categorised 2 of the PCD traces as abnormal.

It is plausible that these results demonstrate a secondary dysfunction of the chloride channel function of CFTR, with either no effect/ or increased inhibition of ENaC. Inflammatory mediators may differentially affect these ion functions causing an uncoupling. This hypothesis is supported by *in-vitro* cell culture studies which showed using immunodetection experiments that IFN-γ lowers CFTR expression and down regulates ENaC, again a disconnect between these two functions [161].

The results are also similar to the studies exploring CFTR dysfunction secondary to cigarette smoke in subjects with COPD, which reported reduced chloride secretion in the nasal and lower airways as evidence of CFTR dysfunction [170, 171]. In both cases, the reduced chloride secretion compared to controls was was not accompanied by hyperpolarized basal measurements. The NPD results were accompanied by a specific reduction in CFTR mRNA expression in nasal curettage

samples obtained from individuals with COPD, and the lower airway traces with suppressed CFTR protein expression in endobronchial biopsy specimens. The authors postulated that cigarette smoke causes a systemic abnormality, producing a widespread pro-inflammatory environment. Of note, in our study, sweat chloride measurements provided a multi-system marker of CFTR activity in the skin. No difference was detected between the sweat chloride concentration of subjects with PCD and controls. This suggests that if there is secondary CFTR dysfunction, it is not systemic, or at least, if systemic effects were present, they were not detectable by this sweat chloride assay.

Alternatively, the reduced chloride response detected in subjects with PCD, coupled with a low baseline and minimal amiloride phase, may reflect a 'blunted' response caused by injury to the epithelial integrity which is a recognised limitation to NPD measurements (cross reference). Patients with PCD are known to manifest more disease in the upper airway than subjects with CF, including chronic rhino-sinusitis and chronic secretory otitis media (ref). Supporting this interpretation are the results from the nasosorption measurements, which are discussed below, and the results from the medical questionnaire. Despite all patients in this study being clinically stable at the visit, the questionnaires revealed that the PCD group suffer from more upper airway symptoms than either CF or controls and are therefore more likely to have chronically damaged epithelium.

In order to determine which of these hypotheses is correct, it would be useful to extend the study to include molecular measurements of the presence of CFTR. This could be achieved by obtaining nasal brushing samples in the same group of subjects and measuring the quantity of CFTR protein using western blotting, and of CFTR mRNA using Taqman assays, although such markers are often insensitive. As inflammatory mediators are thought to modulate the gene expression of CFTR, the expectation would be that CFTR protein levels and CFTR mRNA would be high in controls, reduced in PCD and absent in CF [339]. In addition, it would be helpful to add an extra step when recording the NPD measurements and perfuse ATP onto the epithelium. ATP activates chloride secretion through alternative (non-CFTR) calcium channels and serves as a marker of epithelium integrity [340].

6.5.2 Nasosorption

The results from the nasosorption samples suggest that higher concentrations of inflammatory cytokines are found in the nasal secretions from subjects with PCD than CF and controls. This was mainly as a result of samples from 3 patients, which showed high concentrations across the full

range of cytokines. The results for the other subjects in the PCD groups were comparable to those seen in both CF and control. As nasosorption is a relatively novel technique, there are no studies to date demonstrating that subjects who suffer from chronic rhinosinusitis, bacterial infections or ongoing clinical symptoms have raised inflammatory cytokines in the nasal epithelial lining. However, there is ongoing research in this field (NCT02284074) at Imperial College, London and the results will be informative for this study.

Nasosorption measurements are very variable (CV 27-95%) and therefore difficult to interpret. It is possible that the large variation in the concentration of cytokines detected when comparing samples from different nostrils from the same subject is a true reflection of *in-vivo* conditions caused by localised collections of mucous and secretions. The large variability in the samples taken from the same Eppendorf however questions the validity of these results. The most likely explanation is that, as demonstrated, the concentration of cytokines detected in this study lie at the extreme low end of the optimal dynamic range of the MSD assay. This decreases the sensitivity of the assay and the very small values accentuate the CV% of the measurements. Alternatively, the secretions may not be homogeneously distributed in the saline, due to collections of airway debris and mucous. The methodology for this technique needs to be optimised in order to gain meaningful results. Other studies have used a nose clip during the collection of the nasal secretions to ensure optimal contact between the filter paper and the mucosal surface, which could be included in the methodology. Furthermore, studies have shown that addition of buffer (BSA 1% or Tripton X 1%) prior to elution of the fluid increases the mean recovery of cytokine (10.1136/thx.2010.151043.29-google), and smaller volumes (<500mcl) would reduce the dilution effect of this stage. Following the addition of this step, the repeatability and validity of the samples needs to be confirmed prior to evaluating the difference between both nostrils and comparison of the patient groups.

6.5.3 Relationship between levels of inflammatory mediators and NPD traces

No correlation was revealed between the levels of CFTR function in patients with PCD, as shown by sodium transport (basal PD and amiloride response) and chloride transport (total chloride response) and levels of inflammation. Given the limitations of both measurements as described above, and the small patient size, these results were expected. It would be interesting to re-examine these relationships when the methodology has been optimised.

6.5.4 Limitations

The main limitations of this study are the small number of patients. In addition, NPD measurements were only performed on 1 occasion, and a minimum of 2 sets of values would help determine if the flat traces in the PCD group were seen consistently, reflecting CFTR dysfunction, or were specifically related to a recent, not clinically detectable infection. As mentioned, this study did not include assays for the quantity of CFTR protein and mRNA-CFTR, or microbial culture of the nasal secretions, which would have been useful in the interpretation of both the NPD results and the nasosorption data. Instead symptom questionnaires were used, of which the main disadvantages as outcome measures is that they do not take into account recall bias of patients. This is particularly relevant to patients with chronic conditions such as CF and PCD, as often symptoms have been life long and there is a tendency to under report.

Whilst we measured an array of cytokines, we did not measure other markers of inflammation, in particular NE, which has been shown *in-vivo* and in murine models to degrade CFTR. Finally, refining the methodology of the nasosorption collection and analysis is required, to better understand and interpret these results. This would include the measures described above, use of a nose clip during collection of the secretions, the addition of a buffer prior to the elution of the fluid and adjustment of the volume of the diluent to achieve values for cytokines better in the optimal range of the MSD assay. Repeat measurements on the same subjects would have helped to determine the repeatability of the data.

6.6 Summary and future work

The overarching aim of this study was to establish whether there is acquired impairment of CFTR function manifest by dysregulated chloride and sodium transport in subjects with PCD, and whether this correlates with markers of inflammation. Whilst it is not possible from this data set to determine whether the reduced chloride response demonstrated in the NPD measurements from subjects with PCD; comparable to that seen in CF, is as a result of an acquired local CFTR dysfunction secondary to interaction with inflammatory mediators, or a generalised response to epithelial damage, the work provides a basis from which to further explore CFTR dysfunction in CSLDs other than CF. From the data reported here, it is not possible confidently to confirm or refute my hypothesis that there are acquired deficits in CFTR modulated chloride and sodium transport secondary to local inflammation.

Future work in this field would include better characterising NPD traces in subjects with PCD, by both performing repeat measurements in the same patients, and in a larger patient group, perhaps after treating upper airway infection. The inclusion of molecular measurements of CFTR would help with the interpretation of these results and help in the understanding of the mechanism of

interaction between infective agents and CFTR. Further work is required to optimise the methodology in the collection and analysis of nasosorption, as described above, before it can be utilised as a useful tool to assess upper airway inflammation. It would be useful to process the samples that are stored and measure and compare the levels of NE between each group. Following this, the effect of localised, acute and chronic infection on levels of inflammatory mediators can be established using microbial culture or 16sRNA analysis of nasal secretions.

7 Summary and general discussion

This thesis aimed to explore the relationship between lack of CFTR protein function and markers of disease severity in chronic suppurative lung disease in order to better understand how close the relationship is between the loss of CFTR function and disease pathogenesis. In particular, this work set out to test the hypotheses:

- There is a direct relationship between CFTR function and airway health
- There is a bidirectional relationship between CFTR function and airway inflammation:
    - CFTR deficiency leads to inflammation
    - Inflammation impairs CFTR function

Chapters 4-6 in this thesis have each described a study, which has aimed to further our understanding of the relationship between CFTR function and airway health. This chapter brings together the results from the individual studies and reviews how these have contributed to addressing the hypotheses and to our understanding of the overarching theme.

7.1 Direct relationship between CFTR function and airway health

A better understanding of how measures of CFTR function correlate with airway disease will guide the development of novel therapies which aim to restore CFTR function; to help determine how much correction is necessary to produce a clinical benefit, to measure efficacy of the therapy, and to help establish dosing regimes.

The first part of this thesis set out to show that a direct relationship exists between CFTR function and airway health. The initiation of the gene therapy Multidose Trial which took place when I commenced this MD(Res) in 2012, provided a platform from which to base this section of work. This thesis focused on the group of patients, who in addition to the main trial, also participated in the bronchsocopy subgroup. These patients had LAPD measurements and bronchial biopsies performed to provide functional and molecular evidence of successful gene transfer, and to assess airway inflammation. It was hypothesised that the PD measurements of CFTR ion channel function in the lower airway, where CF disease predominates, would correlate with functional and structural markers of airway health and markers of inflammation in the serum and sputum.
Chapter 3 described the Multidose Trial. It was a phase IIb double blind RCT designed to see whether repeat doses of lipid-mediated gene therapy, delivered to patients with CF results in a

clinical improvement. The primary outcome was relative change in FEV_1; other outcomes were assessed including, radiographic measures of disease status, and measurements of inflammation. This trial reported a significant treatment effect, demonstrating a modest difference between both groups (3.7%) in FEV_1 and a trend towards improvement in secondary outcome measures. These results are encouraging for gene therapy, although the treatment effect was predominantly as a result of deterioration in the placebo group versus stabilization on active treatment, which is discussed in the chapter. Further work in this field now focuses on developing a novel viral vector for the *CFTR* gene, F/HN-SIV, which has been shown in preliminary *in vitro* and animal studies, that it can be delivered repeatedly without loss of gene expression, which can last for the life-span of a mouse (approximately 2 years) following a single dose [287]. This vector requires extensive safety testing prior to progressing to clinical trials.

Chapter 3 provided the context for LAPD-focused study described in Chapter 4. 22 patients in the bronchoscopy subgroup had pre-dosing LAPD measurements for analysis. These results revealed a correlation between the most negative distal basal measurements and FEV_1, but no correlation between basal measurements at the carina or the distal chloride response and other outcome measures. Paired LAPD pre and post treatment traces (n=17) showed a modest, but statistically significant, difference in chloride secretion in the lower airway in the active group compared to placebo (mean difference of 4.4mV). There were no changes in other LAPD parameters, and levels of vector-specific mRNA were below the limits of detection. The change in chloride secretion did not correlate with any of the changes in physiological (including LCI) or inflammatory markers of disease.

These results suggest that there may be a relationship between sodium hyperabsorption (reflecting lack of inhibition of ENaC by defective CFTR) and FEV_1, supporting published literature in which studies have shown a correlation between NPD basal measurements/amiloride response and disease severity. There are several reasons why no direct correlation was revealed between the CFTR-chloride ion transport at baseline and following interventions, and physiological outcome measures of disease status and inflammation. These have been discussed in detail in the relevant chapters, and are outlined below, noting that they are not mutually exclusive.

Limitations on outcome measures

Measures of CFTR function

LAPD measurements had a high variability, both within-measurements and between

measurements in the placebo group (Section 4.6.6); higher than that seen for NPD measurements [190-191], likely reflecting the increased complexity of the procedure in addition to inherent fluctuations in airway physiology. I have shown that the measurements are not sensitive enough to detect small treatment effects unless large numbers of patients are studied, and the number of patients in the bronchoscopy subgroup was too small to reveal subtle correlations. Furthermore LAPD has limitations as an outcome measure for detecting efficacy of topically delivered gene therapy. CF airways have large quantities of mucus and airway secretions; therefore it is likely that gene therapy is unevenly distributed. As LAPD measurements are only performed on a small area of the overall epithelial surface, they might not capture areas that have received the product.

Measures of disease severity

FEV_1 is recognised to be an insensitive disease outcome measure, particularly in subjects with mild lung disease, and the rate of annual decline has decreased [124]. FEV_1 is also thought mainly to reflect disease in the proximal airways, thus may not detect changes in the distal airway. LCI is more sensitive to detect airway damage, and thought to reflect distal airway disease however there are still inherent limitations to the technique. Interventions that cause movement of mucus or airway secretions may result in recruitment of additional lung units not previously contributing to the MBW, or blockage of other lung units, leading to variable changes in the LCI (Section 2.2.3). The bronchoscopy subgroup was small; therefore individual abnormal results could bias the data.

In the main trial, when the patients were stratified into more severe disease (FEV_1=50-69%) and more mild disease (69-89%), the severe group showed a greater treatment effect in FEV_1, whilst the mild group revealed a trend towards improvement in LCI. This may reflect more deposition of gene therapy in the proximal airways of severe patients and more distal in the mild subjects [280]. This highlights that CF is a heterogeneous disease, and particular outcome markers may be more applicable to certain patients.

I described a lack of correlation between CFTR ion channel function and levels of inflammatory mediators in the serum, sputum and endobronchial biopsies; however, these biomarkers also have limitations. Serum inflammatory markers assess systemic inflammation, and whilst often elevated during pulmonary exacerbation, they are usually normal in CF patients during periods of clinical stability; limiting their use as outcome measures for therapeutic studies [215]. Sputum biomarkers of inflammation are increasingly being used to monitor disease activity, however studies examining the relationship between changes in airway inflammation have varied results. Sputum IL-8 and NE in particular have been shown to correlate with FEV_1 in a large cross-sectional analysis [236, 341]

and to be elevated during pulmonary exacerbations [342], whilst other studies have reported only changes in sputum DNA concentrations [343]. Currently it is not known which sputum biomarkers are the most informative, and more research is required on short-term and long-term variability of measurements in CF subjects, and their responses to treatment. Endobronchial biopsies are a relatively novel outcome measure for measurement of airway inflammation. Studies have only been cross sectional and longitudinal studies are required to see how changes progress over time to evaluate its use as an outcome measure. Analysis of biopsies also only measures changes in the proximal airway (accessible by bronchoscope), and not in the peripheral small airways where CF disease is thought to originate.

The effect of gene therapy on the airway

It is possible that the improvement in FEV_1 demonstrated with gene therapy was not due to CFTR restoration itself, but a non-specific reaction to the plasmid complex. Although unlikely, in view of only modest changes in LAPD electrophysiological outcome measures and absence of detection of vector-specific mRNA, this cannot be ruled out. In which case, a correlation between LAPD measures and physiological outcome measures would not be expected.

Underlying physiology

Although CFTR is thought to function predominantly as a chloride ion channel, it is plausible that there is not a direct correlation between chloride function and disease.

- CFTR has more roles than solely controlling chloride secretion. There has been increasing research into the role that the inhibition of control of ENaC, and subsequent sodium hyperabsorption plays in disease pathogenesis [294,294]. Whilst our results from the basal LAPD measurements support this theory, it requires exploration in larger patient groups. As discussed (Section 3.1.1.1), to date no gene therapy trials have reported a change in sodium absorption, as it is though to require almost 100% CFTR correction. Initial studies exploring NPD traces following ivacaftor treatment did not report a change in the parameters reflecting sodium absorption and only chloride transport has been reported. More sensitive measures of sodium transport may be needed to explore this correlation further.

- CFTR is also thought to be expressed on immune cells, which may be integral to their rolein host defence (Section 1.2.3.3). Studies have shown that treatment with ivacaftor *in vivo* and *ex vivo* results in alteration of airway surface markers of inflammation suggesting that their activity is affected by CFTR restoration. This may affect the correlation between airway CFTR ion channel function and disease and inflammation.

- Other factors may also impact on the progression of disease including; infectious status, airway irritants, pancreatic function and modifier genes, including, polymorphism in genes close to *CFTR* and environmental factors. In addition, lower socioeconomic status and reduced adherence to medications has been shown to be associated with worse pulmonary outcomes [344, 345].

The most notable limitation to this work is the small patient group. Whilst this is inherent studying rare diseases, it meant that the study was underpowered to produce significant results. The large variability in the LAPD values means that this measure is too insensitive to draw firm conclusions from, and questions its use as a feasible outcome measure in future trials.

This study did not support the hypothesis that there is a direct relationship between CFTR function and airway health. It is possible that there is a correlation between distal basal LAPD values and FEV_1, indicating the role of ENaC in disease pathogenesis, however this requires further exploration in larger studies. The relationship between CFTR chloride ion transport and disease pathogenesis appears to be complex. Future work in this field requires developing assays for CFTR, which are sensitive and reproducible. In addition, there is ongoing need for more sensitive measures of airway disease, which can be used to monitor patients with CF longitudinally, and response to therapies.

Since this study failed to confirm a direct effect of dysregulated chloride and sodium transport on CF airway disease, in the next chapter I explored the hypothesized bidirectional relationship between CFTR and inflammation. If shown to exist, this may explain the indirect relationship between the two that had been observed.

7.2 There is a bidirectional relationship between CFTR function and airway inflammation

Recent evidence suggests that the relationship between the CFTR protein and inflammation is

complex and bi-directional with suggestions that impaired CFTR function results in a hyperinflammatory response, and that inflammatory mediators may cause secondary CFTR dysfunction. A better understanding of the inflammatory profile in subjects with CF may reveal new therapeutic targets and for both patients with CF and other CSLDs, an acquired defect in CFTR protein function may contribute to disease pathogenesis.

This thesis describes two studies designed to assess these hypotheses in turn. In both studies patients with CF were compared to controls and subjects with PCD. PCD shares similar characteristics, with defective MCC and a similar inflammatory profile to CF, but with presumed normal CFTR function.

7.2.1 Impaired CFTR function leads to a hyper-stimulated inflammatory profile

Chapter 5 describes a study designed to explore whether CF epithelial cells contribute to the high levels of inflammation characteristic of the airway disease, with defective CFTR causing a hyperinflammatory response, at baseline and/or in response to bacterial stimulation. Cells from controls, patients with CF and PCD were cultured at an ALI. 9 pro-inflammatory cytokines in the cell culture supernatant were measured at baseline and after stimulation with respiratory pathogens; *PA, SA* and *HI* at regular time-points. The hypothesis was that CF cells would elicit higher levels of inflammatory mediators at baseline and following bacterial stimulation than non-CF cells. In total only 13 ALIs were cultured (control=5, CF=4, PCD=4), underscoring the complexity of the technique. A comparable inflammatory response was elicited in select cytokines (IL-6, IFN-γ, TNF- α) in all 3 groups, whilst other cytokines, including IL-8, failed to demonstrate a measurable response, not supporting the theory that CFTR dysfunction results in a hyper inflammatory response. This is either due to limitations in the study design, or because impaired CFTR function in epithelial cells does not make them innately hyperinflammatory; outlined below.

<u>Study design</u>

We only achieved 25% success rate in obtaining ciliated ALI samples therefore this study was ultimately underpowered. The levels of the cytokine responses were shown to be very variable in all the groups, and could have been skewed by individual results.
Despite managing to elicit an IL-8 response to bacterial stimulation in the pilot studies, this was not achieved in the main study. The measurements were performed on different ELISA platforms, however they had a comparable optimal dynamic range therefore this result is unexplained. The

methodology needs to be refined in order to address this, as IL-8 is a major pro-inflammatory cytokine present in CF inflammatory airway disease [236].

It would also be useful to measure the levels of NE in the supernatant, as this has both been shown to be elevated in CF airway and to correlate with disease severity [236]. NE has also been implicated in promoting inflammation in the CF airway due to its interaction with epithelial surface molecules involved in the inflammatory pathways (Section 1.2.3.4).

Cell culture

The limitations of cell culture models have been discussed in Chapter 5. They are a useful model to explore cell behaviour *in-vitro* however they do not replicate the complexity of the airway environment or bronchial epithelial cell reactions *in-vivo*. Cell culture removes epithelial cells from the effect of other parts of the immune system such as circulating cells in the blood, which may alter their inflammatory response. Culturing and passaging cells can alter the expression of cell-specific characteristics, such as cell polarity, tight junction formation, mucus secretion and cilia formation. Our results, showing extremely variable inflammatory responses are similar to the published literature [317]. Genetic and environmental factors also likely have an effect on the tissue cultures and influence the inflammatory response. Of note, we used cells cultured from nasal epithelium as a surrogate for the lower airway, for ease of accessibility. These may not accurately reflect the cell behaviour of the lower airway, where different levels of CFTR are expressed [346].

CFTR but not epithelial cells

It is plausible that the CF airway is hyperinflammatory, but that this is not directly as a result of CFTR dysfunction in the epithelial cells. Dysfunctional CFTR may effect other elements of the immune system, including myeloid-derived cells, neutrophils and macrophages [330-333] all of which were not assessed in this study. It is possible that combinations of many of these pathways affect the immune response in patients with CF and contribute, at different stages, to the chronic inflammatory picture characteristic of the disease.

In summary, cells with absent CFTR function, from subjects with CF, when cultured at an ALI, produced a similar level of inflammatory mediators at baseline and following bacterial exposure to cells from patients with PCD and healthy controls. This does not support the hypothesis, that absent CFTR function results in hyperinflammatory response. Future work in this field would initially involve: increasing the number of cell cultures studied, the inclusion of NE in the assays,

and optimising the study design to elicit an IL-8 response. Simultaneous measurements of the inflammatory profile of bronchial and nasal epithelium in the same subjects would evaluate whether nasal cells can be used as a surrogate for the bronchial epithelium. In addition, the measurement of CFTR function in the cell culture specimens using a cell clamp, prior to bacterial stimulation, would allow for comparison of the levels of CFTR ion channel function with the subsequent inflammatory response.

As *in vitro* experiments may not mimic *in vivo* conditions, including cellular and molecular interactions, I went on to study the interactions between CFTR function as demonstrated by nPD and airway inflammation *in vivo*.

7.2.2 Inflammation impairs CFTR function

To date, studies exploring secondary CFTR function have been limited to subjects with COPD. However, it is difficult to disentangle the role of inflammatory mediators and tobacco on the airway epithelium. Chapter 6 describes a study of NPD in subjects with PCD, CF and controls designed to compare CFTR function *in-vivo* with localised inflammatory mediators. The expectation was that the NPD traces in subjects with PCD would show evidence of CFTR dysfunction, which would correlate with increased levels of localised inflammation. Nasal MLF was collected using nasosorption, and the samples were analysed for a range of inflammatory cytokines.

The NPD traces in patients with PCD demonstrated decreased total chloride secretion, comparable to that seen in subjects with CF, however this was not accompanied by a hyper-polarised baseline and exaggerated amiloride response that would be expected with CFTR dysfunction and associated disinhibition of the ENaC channel; in fact, several of the traces demonstrated ‘blunted’ responses suggestive of secondary inflammatory damage. Nasosorption samples revealed elevated levels of inflammation in patients with PCD compared to the other groups (consistent with previous *in vivo* work), however levels of inflammation did not correlate with altered sodium or chloride ion transport. Notably, there was a large variability in the levels of all cytokines measured by nasosorption. These results suggest either an isolated effect of inflammatory mediators on CFTR, uncoupled from ENaC, or may represent damage to the epithelial integrity, a known limitation of NPD measurement. These are discussed.

<u>Isolated effect on CFTR</u>

Previous studies exploring a secondary CFTR defect support that the NPD traces may reflect an isolated CFTR defect. *In-vitro* studies using immunodetection methods have demonstrated that IFN-γ lowers CFTR expression and down regulates ENaC, thus affecting both channels differently, and studies exploring CFTR dysfunction in the lower airways of patients with COPD also revealed reduced chloride secretion but without a hyperpolarised baseline.

Effect of epithelial cell integrity

It is well established that PD measurements are affected by non-specific tissue damage such as exposure to inflammation, which disrupt the epithelial integrity and produce a 'blunted' trace with a lower (less negative) basal value and reduced amiloride and chloride response. These traces are indistinguishable from the traces obtained in the subjects with PCD in whom no deflection was seen on the perfusion of different solutions.

The nasosorption samples revealed higher levels of inflammatory cytokines in MLF obtained from PCD subjects compared to CF and healthy controls, which are consistent with *in-vivo* studies [48,49]. Unfortunately the results were too variable to draw firm conclusions from, although higher levels of inflammatory markers did not correlate with altered levels of sodium or chloride transport. Further work is required to establish whether nasosorption will be a useful tool to explore airway inflammation.

Other tools may be required to assess the link between inflammatory mediators and acquired CFTR dysfunction in CSLD. Previous studies have used techniques to quantify the CFTR protein using immunodetection, and CFTR mRNA with real-time polymerase chain reaction (RT-PCR) TaqMan assays. The main limitation in this study was the small numbers of patients in each group, meaning that it was underpowered. In addition, the techniques utilised to collect the nasosorption samples and analysis need to be optimised in order to determine whether these accurately reflect the *in-vivo* inflammatory profile.

We revealed that subjects with PCD have reduced chloride secretion detected by NPD measurements, however we were not able to conclude that this was due to the effect of inflammatory mediators on CFTR function. Further studies will involve additional assays to detect and quantify the CFTR protein to help determine the cause of the abnormal NPD traces.

Combined, these studies do not provide evidence that there is a bidirectional relationship between CFTR and inflammation, but provide a basis upon which to base future studies to try and better understand how CFTR interacts with its environment. This will be beneficial to both patients with CF and CSLDs, and may reveal potential therapeutic targets. The development of animal models of CF, and advances in cell-modeling tools such as organoids may facilitate this research.

7.3 Overall conclusion

When it was first discovered in 1989 that patients with CF had a defect in the gene encoding for the CFTR protein, and the subsequent elucidation of this protein predominantly as a chloride channel, it was thought that a treatment for CF would rapidly follow. This did not prove to be case, as it was increasingly understood that the pathogenesis of CF airway disease is complex. Despite this, in the last decade there have been phenomenal advances in CF care and resultant survival in patients with CF. This thesis described a trial of gene therapy in patients with CF, demonstrating promising results, in addition to the advancing field of CFTR modulators, such as ivacaftor, which are beginning to change the clinical course of patients with CF, albeit currently only with select mutations.

This thesis has set out to explore the relationship between CFTR function and airway disease in both CF and other CSLDs. The findings support the theory that it is not the chloride function of CFTR in the airway epithelium that is solely responsible for disease progression and that other consequences of CFTR dysfunction play a role. This thesis provides the basis for more studies to explore these complex interactions, with the hope that this will help guide the development of future therapies for CF.

8 References

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9 Appendices

Appendix A: Formulation of nasal and lower airway potential difference perfusate solutions

Solution 1: Ringer's (Basal) Solution

Compound			Concentration	
			mM/L	g/L
Sodium Chloride		NaCl	135	7.83
Calcium Chloride Dihydrate		$CaCl_2$-$2H_2O$	2.25	0.33
Magnesium Chloride Hexahydrate		$MgCl_2$-$6H_2O$	1.2	0.24
Monobasic Potassium Phosphate		K_2HPO_4	2.4	0.42
Dibasic Potassium Anhydrous		KH_2PO_4	0.4	0.05

Solution 2: Zero Chloride Solution

Compound			Concentration	
			mM/L	g/L
Sodium Gluconate		$C_6H_{11}NaO_7$	135	29.43
Calcium Gluconate Anhydrous		$C_{12}H_{22}CaO_{14}$	2.22	0.95
Monobasic Potassium Phosphate		K_2HPO_4USP	2.4	0.42
Dibasic Potassium Phosphate Anyhydrous		KH_2PO_4	0.4	0.24
Magnesium Sulphate $7H_2O$		$MgSO_4$-$7H_2O$	1.2	0.3

Ringer's + Amiloride (0.1 mM) Solution (RA)

Amiloride 0.03 mg/ml (0.1 mM) is added to Ringer's Solution (Solution 1)

Zero Chloride + Amiloride (0.1 mM) Solution (ZCl/A)

Amiloride 0.03 mg/ml (0.1 mM) is dissolved in a ZCl Solution (Solution 2)

This solution is used for the measurement of nasal PD

Isoprenaline (0.01 mM) containing Solutions

To all solutions containing isoprenaline (listed below), isoprenaline hydrochloride (1mg/5ml) 0.625 ml (=0.125 mg) is added to 50 ml of 'base' solution and agitated

Isoprenaline must be added just prior to use, owing to expiry time of 2 hours from the time dissolved

Zero Chloride + Amiloride (0.1 mM) + Isoprenaline (0.01 mM) Solution (ZCl/A/Iso)

Isoprenaline dissolved in ZCl/A solution.

This solution is used for measurements of nasal PD

Zero Chloride + Isoprenaline (0.01 mM) Solution (ZCl/A/Iso)

Isoprenaline dissolved in ZCl solution.

This solution is used for measurements of lower airway PD. It does not contain amiloride.

Appendix B: Patient assessment for fitness to dose

A clinical assessment was made by either of the trial Doctors (myself or Dr Michael Waller) on the fitness of the patient to dose, ensuring the patient had not deviated outside pre-defined criteria and assessing for relative clinical stability

- Percentage predicted FEV_1 >40%
- Fall in FEV_1 <20% from the last visit (Pre-dosing)
- Patients' body temperature <37.7oC
- Patients' oxygen saturation >94%
- No new abnormality identified at clinical examination
- Not commenced iv antibiotics within the last 7 days
- Not taken nebulised rhDNase (pulmozyme) within the past 24 hours

If patients had fallen outside these dosing criteria the dose could be deferred to a new date within the patient's dosing window (28 + 5 days) to prevent missed doses, otherwise the dose was forfeited (a maximum of 3 missed dose being allowed during the study)