The behaviour of the cystic fibrosis respiratory epithelium and its response to multidose *CFTR* gene therapy

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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.

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Abstract

Cystic fibrosis (CF) is a clinical syndrome resulting from inherited mutations to the cystic fibrosis transmembrane conductance regulator (CFTR) protein, whose absent or reduced function results in abnormal epithelial ion transport and an abnormal transepithelial potential difference (PD), leading to downstream epithelial dysfunction and a pathognomonic clinical phenotype. Most treatments to date manage the disease sequelae of airway mucus and infection, but correction of the underlying defect for all patients is the ultimate aim. Gene therapy offers the potential as a universal treatment to ameliorate CFTR function, leading to clinical benefit.

This thesis will centre on the recently completed Multidose Trial (MDT) – the repeated application of non-viral *CFTR* gene therapy in patients with cystic fibrosis. The trial was undertaken during the entirely of this PhD, and recruited 136 patients (aged ≥12) with CF, and randomised to receive 12, monthly nebulised doses of a cationic-lipid (pGM169/GL67A), or placebo. The hypothesis of the study was that repeated administration of non-viral gene therapy would produce vector-specific epithelial CFTR, leading to demonstrable improvements in lung function and measureable *de novo* chloride ion transport in the upper (nasal) and lower (bronchial) airway. The study reports stability in lung function with gene therapy (n=62) at 48 weeks compared to a decline in the placebo group (n=54), concluding a significant treatment effect of a relative improvement of 3.7% (p=0.046) in percent-predicted forced expiratory volume in 1 second (FEV_1) at follow-up; other clinical parameters further support a treatment benefit, however a reduction in the frequency of pulmonary exacerbations was not detected. A *post hoc* analysis identified a significant treatment benefit of 6.4% in patients with more severe airways disease ($ppFEV_1$ 50-70%), however failed to detect a treatment effect in patients with less severe disease ($ppFEV_1$ 70-90%). Individual patients demonstrated *de novo* chloride transport in the nasal and lower airway, as measured by epithelial potential difference, with a significant difference being measured in the lower airway in

response to gene therapy (-4.4 mV, p=0.03); no difference was identified in the upper airway in response to active treatment.

The thesis will next explore the relationships of epithelial PD between the nasal and lower airway with measurements of lung function at baseline, and in response to treatment with gene therapy. At baseline, trends between sodium transport in the nasal epithelium and FEV_1 and a lung clearance index (LCI) (p=0.01) were identified; no relationship was identified between chloride indices, or with any measurement from the lower airway. No relationship between the electrophysiology of the upper and lower airway epithelium was detected.

The author will present a novel method used to maintain blinding whilst performing nasal PD (NPD) measurements during the MDT, using a 2-operator technique. This technique is reported as not inferior to the standard NPD method and supports its validity of its use for future studies, but provides caution that this method may take longer to perform and that more data may be excluded owing to poor NPD trace quality.

The thesis concludes by describing two studies designed to further understand the performance and interpretation of PD measurements, and discussing the overall usefulness of airway PD as a clinical trial outcome. The first study investigates the amount of total chloride secretion (in healthy (non-CF) volunteers (n=18)) in the nasal epithelium by perfusing 'standard nasal' (with amiloride) and 'lower airway' (sans amiloride) solutions, reporting that more (approximately 50%) chloride is secreted in the presence of amiloride-containing solutions, and when the duration of perfusion was extended.

The final study aimed to define the minimum period for performing sequential NPDs in the same (CF) patient, reporting that 4/8 (50%) CF patients basal PD had returned after 30 min, and that basal PD had returned by 60 min in all patients, concluding that repeated measurements could be made over a short timeframe for clinical and research studies.

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Abbreviations

Abbreviation for clinical studies

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
The Multidose Trial (MDT)	A randomised, double-blind, placebo-controlled Phase 2B clinical trial of repeated application of gene therapy in patients with cystic fibrosis

Abbreviations used throughout this thesis

AE	Adverse events
Ag/AgCl	Silver/Silver chloride (electrode)
ASL	Airway surface liquid
ATP	Adenosine triphosphate
ATS	American Thoracic Society
BAL	Bronchioalveolar lavage
BMI	Body mass index
BP	Blood pressure
CaCC	Calcium-activated chloride channels
cAMP	Cyclic adenosine monophosphate
CBAVD	Congenital bilateral absence of the vas deferens
CEV	Cumulative expired volume
CF	Cystic fibrosis
CFQ-R	Cystic fibrosis questionnaire - revised

CFRD	CF-related diabetes
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
DSMB	Data and Safety Monitoring Board
ECSF	European Cystic Fibrosis Society
EMA	European Medicines Agency
ENaC	Epithelial sodium channel
ERS	European Respiratory Society
FDA	U.S. Food and Drug Administration
FEV_1	Forced expiratory volume in 1 second
FRC	Functional residual capacity
FVC	Forced vital capacity
GA	General anaesthesia
GL67A	Cationic lipid used in clinical programme
GTAC	Gene Therapy Advisory Committee
HR	Heart rate
HRCT	High-resolution computerised tomography
ICM	Intestinal current measurement
ICS	Inhaled corticosteroid
IL	Interleukin
IMP	Investigational Medicinal Product
IQR	Interquatile Range
IRT	Immuno-reactive trypsinogen
ITT	Intention to treat group
IV	Intravenous
LAPD	Lower airway potential difference

LCI	Lung Clearance Index
MBW	Multiple breath washout
MCC	Mucociliary clearance
MCID	Minimally clinically important difference
MDT	Multidose Trial
MHRA	Medicines and Healthcare products Regulatory Agency
MMP	Matrix myeloperoxidase
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MSD	Membrane spanning domain
NF-kB	Nuclear factor-kB
NICE	National Institute of Health and Care Excellence
NMD	Nucleotide-binding domains
NPD	Nasal potential difference
NRES	National Research Ethics Service
PBS	Phosphate buffered saline
PBS	Phosphate-buffered saline
PCL	Periciliary liquid
PCR	Polymerase chain reaction
PD	Potential difference
PE	Pulmonary exacerbation
pGM169	*CFTR* plasmid used in clinical programme
PIC	Patient identification centre
$ppFEV_1$	Percentage predicted forced expiratory volume in 1 second
PTC	Premature termination codon
QOL	Quality of life
rAAV	Recombinant *Adeno-associated virus*
rAd	Recombinant *Adenovirus*
RR	Respiratory rate
RSV	Respiratory syncitial virus
SAE	Serious adverse event
SD	Standard deviation
SOP	Standard operating procedures

SpO_2	Oxygen saturations
TNF	Tumour necrosis factor
UKCFGTC	UK Cystic Fibrosis Gene Therapy Consortium
URTI	Upper respiratory tract infection
WCC	White blood cell count

Abbreviations used within this thesis for potential difference perfusion solutions

Ringer's	Ringer's solution
RA	Ringer's solution with amiloride (0.1 mM)
ZCA	Zero Chloride Solution with Amiloride (0.1 mM)
ZCAI	Zero Chloride Solution with Amiloride (0.1 mM) and Isoprenaline (10 μM)
ZCI	Zero Chloride Solution and Isoprenaline (10 μM)
tCl^-	Total chloride response

Abbreviations for the medical and research team at the Royal Brompton Hospital (including the author)

MDW	Dr Michael Waller (author)
EA	Professor Eric Alton
JCD	Professor Jane Davies
KH	Dr Katharine Harman
CS	Mrs Clare Saunders
NS	Dr Nicholas Simmonds

Abbreviations used within this thesis to identify clinical sites

RBH	Royal Brompton Hospital, London
WGH	Western General Hospital, Edinburgh
RHSC	Royal Hospital for Sick Children, Edinburgh

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Imperial College London	Professor Eric Alton
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University of Oxford	Dr Deborah Gill
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Chapter 1. Introduction

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is a multisystem, monogenic disease predominantly affecting Caucasian populations, and which, despite an increasing emergence of new treatments and therapies remains a life-shortening condition.

CF is caused by mutations in the Cystic Fibrosis transmembrane conductance regulator (*CFTR*) gene, which is inherited in an autosomal recessive manner and thus requires both parents to pass one mutated gene to their affected child [1]; the parents, being heterozygotes ("carriers") for *CFTR*, are unaffected by the disease. 1 in 25 people in the UK are carriers of the faulty *CFTR* gene, and it is estimated that in the UK CF occurs in 1 in 2500 live births.

Treatments to date have considerably improved morbidity and increased life expectancy. Data from UK CF registry data compiled by the CF Trust (2014) records over 10,500 patients currently living with CF – almost 60% of patients are over 16 years old, with 800 people currently alive at over 40 years old [2]. Epidemiological modelling predicts that individuals born in the year 2000 will have a median survival of >50 years old [3], and with modern therapies aiming to correct the underlying cellular defect, exponential increases in life expectancy are a realistic potential.

1.2 Cystic Fibrosis Transmembrane Conductance Regulator Protein

Mutations in the *CFTR* gene result in defects in the CFTR protein, an ATP-binding cassette (ABC) transporter located on the apical surface of epithelial cells within specific organ tissues (sweat glands, conducting airway, pancreatic and biliary ducts, intestinal mucosa and the male vas deferens) [4]. Wild-type CFTR is formed of 1480 amino acids and is composed of 2 membrane spanning domains (MSD) (each made from 6 transmembrane segments) together forming an ion channel, 2 nucleotide-binding domains (NBD 1 and 2) responsible for binding

and hydrolysis of ATP, and a regulatory (R) domain regulating CFTR channel opening [5] (Figure 1.1).

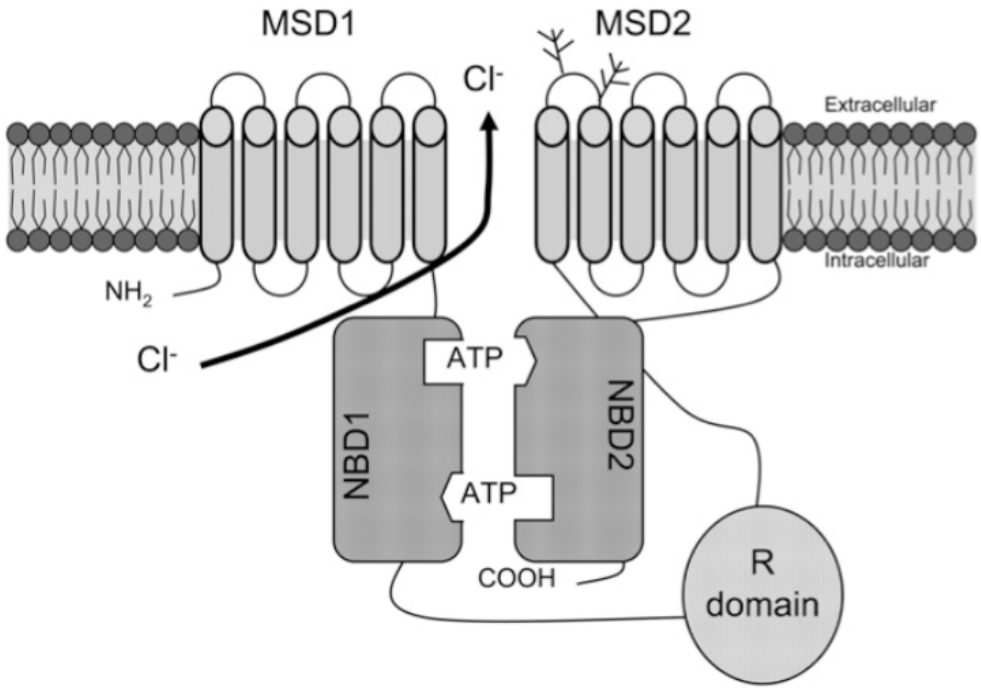


Figure 1.1. Proposed structure of the CFTR protein, reproduced from Rogan *et al* [6]. MSD – membrane spanning domain; NBD – nucleotide-binding domain; R (regulatory) domain; Cl^- – represents a chloride ion.

CFTR functions as an epithelial cyclic adenosine monophosphate (cAMP)-dependent ion channel and is directly responsible for the efflux of chloride and bicarbonate ions from the cell [7-9], with further regulatory roles on the epithelial sodium channel (ENaC) and chloride/bicarbonate exchangers [10, 11]. Wild-type CFTR has 3 states: open, closed, and open-ready, with its activity being regulated by protein kinase A (PKA)-phosphorylation of the R-domain and the presence of intracellular ATP [12]. It is defects in the quantity of CFTR at the apical membrane or impaired channel function that result in pathological epithelial function leading to the clinical features of CF.

1.3 The *CFTR* Gene

The gene encoding the CFTR protein was discovered in 1989 [4] and is located on the long arm of chromosome 7 at location q31.2. Mutations in *CFTR* produce absent, deficient or defective levels of CFTR at the epithelial surface.

1.3.1 *CFTR* class mutations

Disease causing *CFTR* mutations can be divided into 6 distinct 'classes' based on the mechanism of defect in CFTR protein synthesis, the mode of CFTR protein dysfunction or CFTR stability [13]. Mutation classes I – III have a propensity to a more severe disease phenotype owing to the absence in production of any active CFTR protein; classes IV – VI are usually clinically milder and associated with a better clinical outcome owing to residual CFTR function [14].

1.3.1.1 *CFTR* class I – II mutations

Mutations in *CFTR*, such as those possessing a premature stop codon (nonsense), frameshift or aberrant splicing, that result in the production of unstable mRNA and a truncated, non-functional CFTR protein are termed class I mutations. Class II mutations result in defective peptide glycosylation and folding within the endoplasmic reticulum and are thus targeted for proteasome-mediated degradation; their reduced trafficking to the cell surface membrane result in the absence of CFTR on the cell surface membrane. The commonest *CFTR* mutation *F508del* (*phe508del*), present on at least one allele in around 90% of CF patients [15], is an example of a class II mutation. Deletion of phenylalanine at residue 508 (located within the NBM1), produces a protein with defective folding and stability within the endoplasmic reticulum, and is thus degraded within the cytoplasm, considerably reducing functional CFTR in the epithelium cell surface [16].

1.3.1.2 *CFTR* class III –VI mutations

Mutation classes III – VI all produce a full length CFTR protein that is trafficked and incorporated into the apical membrane: classes III and IV both have attenuated CFTR function, whilst classes V and VI have reduced CFTR number or residence time at the membrane. Impaired channel regulation ('gating') (class III) results from the resistance of CFTR to phosphorylation or ATP binding at the NBD, which reduces channel opening and ion transport. The commonest class III mutation is the *G551D* (*Gly551Asp*), a substitution of the amino acid glycine for aspartic acid at residue 551. Class IV mutations are mostly located within the MSD of the CFTR protein and result from impaired channel conductance and a reduced chloride transport through the cell membrane. The final 2 mutation classes both represent CFTR with normal function at the cell surface but in reduced quantity, by either a reduction in synthesis from the combination of aberrant and normal *CFTR* splicing (class V), or reduced CFTR stability and hence increased protein turnover (class VI). A diagrammatic summary of *CFTR* class mutations with examples of disease-causing mutations is shown is Figure 1.2 [17].

To date >2000 mutations in the *CFTR* gene have been identified and geographical variations exist, however not all of these account for the disease phenotype [18]. 97.2% of the patients with CF living in the UK have to date have been genotype; 8.9% of these patients however have at least one unknown allele [19].

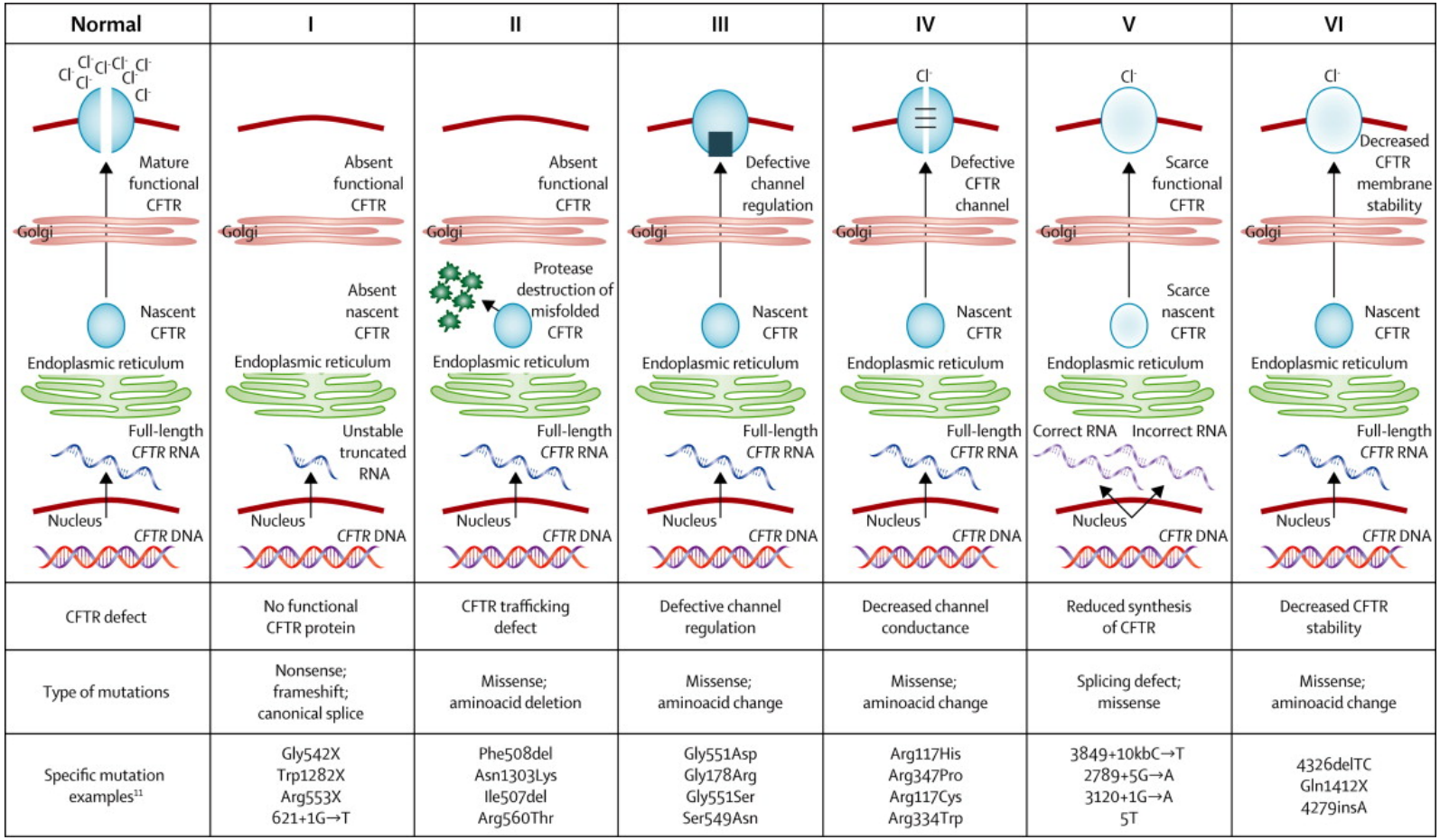


Figure 1.2. Summary diagram showing classes of CFTR mutations, with examples of disease-causing mutations. Reproduced from Boyle and De Boeck, 2013 [17].

1.4 The Role of CFTR in the Healthy Airway Epithelium

CFTR is expressed throughout healthy airway and surface epithelium and in parts of submucosal glands [20], being responsible for direct efflux of Cl^- and HCO_3^- ions, the indirect absorption of Na^+ (via ENaC), and the associated transport of water across the apical membrane. The airway surface liquid (ASL) is a thin fluid layer produced above the airway epithelium and is the interface between the airspace and epithelium – it is comprised of a layer of periciliary liquid (PCL), secreted by airway cells and submucosal glands, which surrounds cilia, and a layer of mucus [21]. It is crucial that the composition of electrolytes, pH and volume of the PCL are maintained to provide an optimal environment for innate defence mechanisms of the airway epithelium by: (i) creating a physical barrier to pathogens and trapping foreign particles, and when appropriately hydrated allowing ciliary beating and by mucociliary clearance (MCC) escalating pathogens away from the airways towards the oropharynx; (ii) bacterial killing by endogenous antimicrobial factors (e.g. lactoferrin and lysozyme) and defensins, and attracting neutrophils and macrophages to the airway surface [22, 23].

1.4.1 The airway surface environment in the CF lung

The ASL in the CF lung is low volume, likely abnormal in ion concentration, and contains thick and sticky mucus; various hypotheses are proposed for these differences.

1.4.1.1 Dehydration of the airway surface liquid

The 'low volume' hypothesis proposes a mechanism for ASL dehydration. In the absence of a functional CFTR protein, its normal inhibitory effect on ENaC is lost leading to the hyperabsorption of Na^+ through the ENaC channel [24]; Na^+ ions are further transported out of the cell (in exchange for K^+ ions) through the Na^+/K^+-ATPase on the basolateral surface [21]. This net movement of Na^+ across

the membrane draws water with it and results in low volume of the PCL and increased viscosity of the mucus layer. This environment does not allow normal ciliary action and leads to failure of mechanical clearance of mucus.

In vitro measurement of the ASL height under static conditions have been demonstrated to be lower in CF epithelium (~3 μm) when compared to non-CF (~7 μm – the height of extended cilia), with the CF model displaying collapsed cilia and reduced MCC of particulate matter [24] (Figure 1.3). However, when repeated with airway cells experiencing shear-stress mimicking conditions of phasic motion of the lung, increases in levels of ATP in the PCL were noted and ASL volume in the CF epithelium was restored to normal levels (~7 μm) being thus sufficient for MCC [25]. It is therefore likely that other mechanisms may interrupt this pathway and downstream ASL production, including disruption to the ATP signalling pathways and insults from infections, especially respiratory viruses. The respiratory syncitial virus (RSV) has been shown to directly (in the absence of cytokines) deplete PCL volume through up-regulation of extracellular ATPase and produce mucus plugging that leads to bacterial infections (via cytokine release and cytotoxic damage to cell cilia).

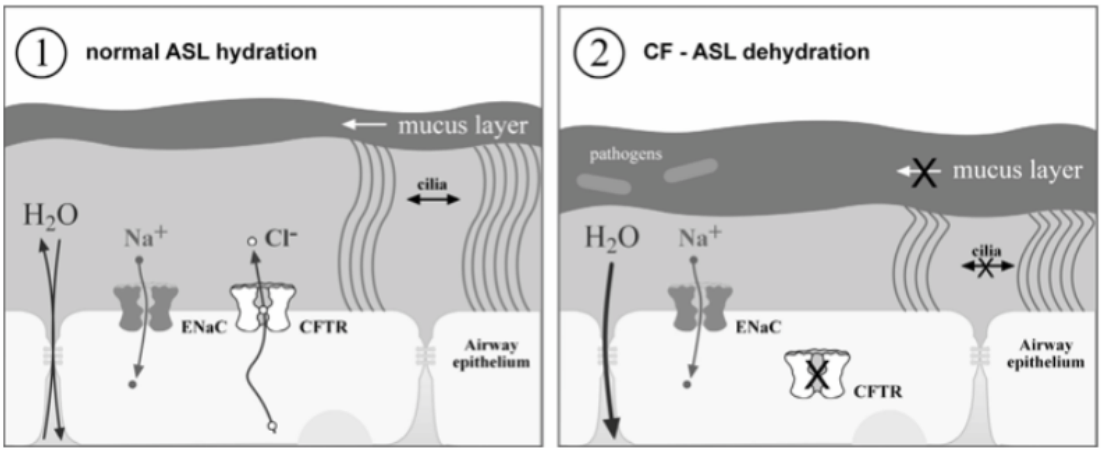


Figure 1.3. Diagrammatic representation summarising (1) normal hydration of the ASL and (2) the pathological changes occurring in the ASL in the CF airway. Reproduced from Althaus, 2013 [26].

1.4.1.2 Reduction in the pH of the airway surface liquid

It is also hypothesised that electrolyte deficiency occurs within the ASL owing directly to absent CFTR channels, specifically reducing levels of HCO_3^-. A lower ASL pH has been demonstrated in CF compared to non-CF airway epithelium in human bronchial epithelial cells [27] and tracheal epithelial cells from CF pigs [28], with the loss of CFTR-mediated HCO_3^- secretion being proposed; *in vivo* studies of CF patients have however failed to conclusively support this difference when compared to the ASL pH of non-CF individuals [29, 30]. It is hypothesised that a reduction in ASL pH and HCO_3^- could result in 2 pathological processes: (i) impairment of the innate immune response, and (ii) impaired mucin expansion leading to viscous mucus.

1.4.1.3 Impairment of the innate immune response

Naturally occurring antimicrobial peptides of the innate immune system, including lactoferrin, lysozyme, surfactant protein-A (SP-A) and cathelicidin, are known to be exquisitely pH sensitive, with conformational changes and reduced bacterial killing demonstrated in the laboratory environment at low pH [31]. A reduced innate immune response has been demonstrated in a CF pig model despite no difference being measured in the abundance of these antimicrobial peptides, and when ASL pH was increased, bacterial killing was restored [28]. These data would support a relationship between the pH of the ASL in CF and an impaired antimicrobial effect.

1.4.1.4 Abnormal mucus within the CF airway

Tenacious airway secretions with reduced MCC cause mucus retention and plugging within the airways, which in turn serves as a nidus for airway infection. Several hypotheses, which are not mutually exclusive, exist to help provide explanation for the abnormally thick mucus seen in CF.

One mechanism proposed is, as described above, the dehydration of mucus at the epithelial surface, as a consequence of the under-hydrated ASL [32]. A second hypothesis relates to the inherent structure of mucin, which is stored as a compacted molecule (a mucin 'granule'), highly concentrated with Ca^{2+} ions which form electrostatic bridges and stabilise the molecule. It is proposed that in the healthy airway, the presence of negatively-charged HCO_3^- cause massive expansion (up to 1000 times) within 1-2 s of secretion of the mucin molecule. It is therefore postulated that in the CF airway lacking in HCO_3^-, mucin expansion cannot occur (Figure 1.4) [8].

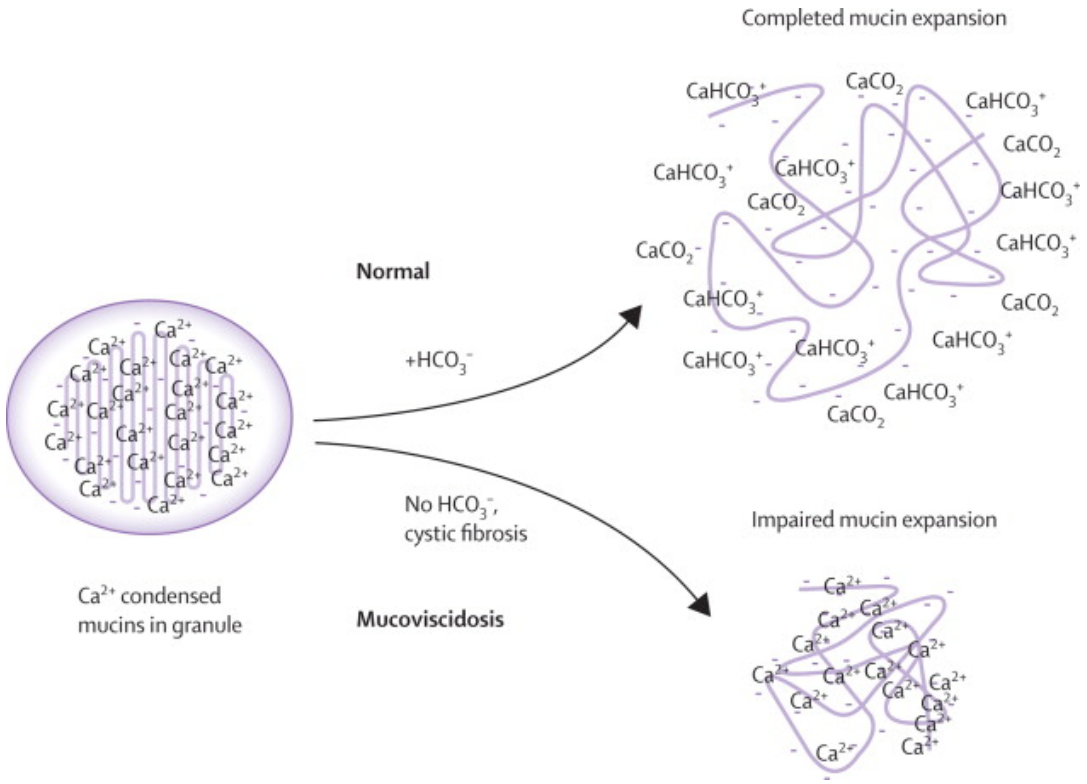


Figure 1.4. Proposed mechanism of the normal expansion of mucin in the presence of $HCO_3^-$and impaired mucin expansion in cystic fibrosis. Reproduced from Quinton, 2008 [8].

1.4.1.5 Bacterial infection in the CF airway

Acute and chronic airway infection is major feature of CF lung disease and a significant cause of morbidity and accelerated mortality, with persistent infection causing chronic respiratory symptoms including cough and sputum production. In part, airway infection relates to the pathophysiological changes reduced innate immunity, reduced MCC, thickened mucus and possibly, impaired killing by soluble defence molecules.

Using traditional culture methods, a relatively small set of bacterial species are commonly identified from the CF airway including *Haemophilus influenzae, Staphylococcus auerus* and *Pseudomonas aeruginosa* [33]; new emerging pathogens are increasing in frequency, probably due to antibiotic pressures. Whilst the incidence of these pathogens varies throughout the life-course of the CF patient, *P.aeruginosa* remains the commonest pathogen infecting the airways of CF patients, although it is suggested that its prevalence may be decreasing [33]. According to recent data published by the UK CF Trust, 63.6% of adult patients (>16 years) were chronically or intermittently infected with *P.aeruginosa* from sputum specimens collected in 2014 [2]. Once chronic infection is established, an increased rate of deterioration in lung function is seen [34, 35], with an increased frequency of respiratory exacerbations, hospitalisation, and an 8-year risk of death 2.6-times higher than patients not colonised by *PA* [36].

It is recognised that 20-30% of bacterial species can be difficult to culture by standard laboratory methods [37]. Culture-independent techniques involve polymerase chain reaction (PCR) amplification of the bacterial 16S ribosomal RNA (rRNA) gene, which possesses nine hyper-variable regions; this facilitates genus and species identification, and estimates entire microbial communities or "microbiome" [38]. Culture-independent DNA-based profiling of CF respiratory secretions has uncovered a further layer of CF microbiology over and above 'traditional' pathogens, yet their clinical relevance remains of unknown. However, it may explain why some CF patients fail to respond clinically to

standard antibiotic therapy whilst others appear to have lung inflammation in the absence of the known pathogens [39].

1.4.1.6 Infection and inflammation of the CF airway

Chronic inflammation of the airways, secondary to infection, is central to the progression of CF lung disease and leads to structural airway damage (bronchiectasis) and progressive airways obstruction [40]. Bacteria, viruses, and fungi trigger an inflammatory cascade in the airway, via the activation of nuclear factor-κB (NF-κB) resulting in the synthesis of cytokines (such as tumour necrosis factor (TNF)-α and interleukin (IL)- 1β, IL-6 and IL-8) [41]. The cytokine response, along with macrophage produce leukotriene B_4 (LTB_4), mediate neutrophil infiltration into the airways as a response to pathogens, yet their activation causes the release of oxidants and proteases causing direct tissue degradation and bronchiectasis [42, 43].

It has been hypothesised that CFTR may play a central role in normal cytokine secretion, although the mechanism between the absence of CFTR and an enhanced inflammatory response remains to be defined [44]. Pharmacological inhibition of CFTR or inhibition of its production with oligonucleotides, results in an increase in the activation of NK-κB and increased IL-8 secretion demonstrating absence of CFTR activity is essential to the inflammatory cascade seen within the CF lung [45].

Significant correlations have been made between raised inflammatory markers measured in sputum and bronchioalveolar lavage fluid (BAL) (in particular neutrophil count, neutrophil elastase, matrix myeloperoxidase (MMP)-9, and IL-8) and worsening lung function and disease severity [46, 47]; in response to antibiotic therapy, these inflammatory markers have been demonstrated to improve and correlate with spirometric measures of lung function [48]. Whilst chronic inflammatory is suggested to be harmful to the airways, it is possible that limiting its effect is also detrimental – a study blocking LTB_4-receptors

reported significant increases in serious adverse events and pulmonary exacerbations [49], proposing a protective role of the inflammatory response.

1.5 The Clinical Syndrome of Cystic Fibrosis

1.5.1 Clinical manifestations of CF

CF is multi-system disease, affecting organs expressing CFTR. Failure of CFTR protein function produces pathognomonic features of abnormal ion transport and lead to the clinical syndrome typified by thickened luminal secretions. It is however the consequences in the lower airway that is the predominant feature of the condition, leading to premature death in approximately 85% of patients [50].

The process of thick ductal secretions may be extrapolated to explain the extra-pulmonary manifestations of the disease with the end-organ failure, namely pancreatic ductal obstruction leading to exocrine failure (pancreatic insufficiency, present in at least 85% of patients [51]) and pancreatic endocrine failure (CF-related diabetes), hepatic ductal obstruction with cholestatic liver disease and cirrhosis, distal intestinal obstruction, and congenital bilateral absence of the vas deferens (CBAVD). The main clinical features of CF within each affected organs are summarised in Table 1.1.

ORGAN AFFECTED	CLINICAL FEATURES
Lower Airway	• Increased, thickened mucus production • Acute & chronic airway infection – especially with *Pseudomonas aeruginosa, Staphylococcus aureus & Haemophilus influenzae* • Atypical Mycobacterial infection • Allergic bronchopulmonary Aspergillosis • Bronchiectasis • Spontaneous pneumothorax • Haemoptysis • Respiratory failure
Upper Airway	• Sinusitis • Nasal Polyps
Pancreas	• Pancreatic insufficiency (exocrine failure) and malabsorption • Vitamin deficiency states • CF-related diabetes (endocrine deficiency) • Recurrent pancreatitis
Hepato-biliary System	• CF-related liver disease (encompassing cholestatic cirrhosis & liver failure) • Biliary stasis and cholelethiasis
Gastro-intestinal Tract	• Gastro-oesophageal reflux • Constipation • Distal intestinal obstruction syndrome • Meconium ileus • Intussusception • Increased risk of malignancy
Genito-urinary Tract	• Male infertility (CBAVD) • Reduced female fertility (thickened cervical mucus) • Delayed puberty (probably secondary to nutritional, inflammatory and/ or drug effects rather than primary) • Stress incontinence

Skin	• Raised sweat chloride (diagnostic gold standard) • Electrolyte depletion (pseudo-Bartter's syndrome) • Vasculitis
Bones	• Low bone mineral density and osteoporosis • CF Arthropathy

Table 1.1. Table summarising organ and system specific clinical features in CF.

1.5.2 Pulmonary exacerbations

A consensus for the definition of a pulmonary exacerbation is yet to be made [52], however clinically it is defined by an episode combining clinical symptoms and parameters: an increase in pulmonary symptoms such as cough, sputum production, wheeze, breathlessness, associated constitutional symptoms, a fall from baseline lung function and elevated haematological markers of infection.

It remains unclear as to what triggers an exacerbation however it is suggested to result from a complex response to microbiological and host defence mechanisms on sputum production and airway obstruction [53]. It is possible that respiratory viruses may play a role, as has been demonstrated in CF children with an RSV infection [54].

A relationship exists between the severity of lung disease and the frequency of pulmonary exacerbations, with fewer exacerbations occurring in patients with a better lung function; furthermore, an increased frequency of exacerbations are met with a failure to fully regain lung function and thus an increased rate in its decline [53].

It is therefore imperative to eradicate new pathogens early preventing chronic airway infection, and then suppressing chronic infection when it ultimately takes hold [55].

1.6 Assessment of Lung Disease in CF

This section will present methods used in clinical and research practice to measure and monitor airway physiology and structural changes within the lung. It will describe two measurements of lung function (spirometry and lung clearance index), measurement of transepithelial potential difference of the airway, and the role of computerised tomography imaging.

1.6.1 Spirometry

Spirometry is defined as "a physiological test that measures how an individual inhales or exhales volumes of air as a function of time" [56]. It is a measurement used throughout all lung disease and offers biometrics in terms of patterns, progression and severity of airways disease. In CF, spirometric assessment of lung function is an important clinical tool, and is used (i) longitudinally monitor disease, being traditionally linked with disease severity, morbidity and mortality [57, 58] (see section 1.8); (ii) to inform clinical decisions relating to airway infection, inflammation or mucus, and responses to treatment [59].

Spirometry is a reproducible measure, performed by the subject making forced breathing manoeuvres, via a mouthpiece, into a mechanical or computerised device calibrated to measure volume and airflow. Spirometry is able to measure many parameters of lung function and is used to highlight abnormalities in different 'compartments' of the airways. The test should be performed by an experienced operator following standardised guidelines produced by the American Thoracic Society (ATS) and European Respiratory Society (ERS) [56].

The most important measures of lung function (both expressed as a volume in litres) that will be discussed within this thesis [56]:

(i) **Forced expiratory volume in 1 second (FEV_1)** – "The maximal volume of air exhaled in the first second of a forced expiration from a position of full inspiration". In CF, inflammation and mucus

obstruction of the larger airways cause airflow limitation, which reduces the volume of air expelled (in 1 sec) after a forced manoeuvre.

(ii) Forced vital capacity (FVC) – "The maximal volume of air exhaled with maximally forced effort from a maximal inspiration"

Volumes obtained are compared to age, sex, height and ethnicity matched equivalents derived from epidemiological data collection and modeling, and percentage predicted values derived (i.e. percentage predicted FEV_1 ($ppFEV_1$)). Two endorsed formulae are those from Wang *et al*, specific to children (aged 6 to 18 years) [60], and that from the Third National Health and Nutritional Survey (NHANES III) from Hankinson *et al* [61], yet a significant limitation in these formulae is the transition from adolescence to adulthood, with absence of continuous modeling across age ranges and the complexities of modeling relating to rapid changes in adolescent growth. Artificial differences in percent predicted lung function without real changes in volumes results if these two formulae are used longitudinally. Further modeling by Stanojevic *et al* addressed these discrepancies, using biologically plausible and continuous references ranges across these age groups [62].

1.6.2 Lung clearance index

Lung clearance index (LCI) is a reliable, validated assay that provides measurements of global ventilation inhomogeneity throughout the lung, including from the small airways that are affected in the early stages of CF lung disease but is poorly detected by spirometry largely reflecting proximal airway [63]. LCI can thus be useful clinically in CF disease for young children or patients with early/mild lung disease where the FEV_1 remains within the normal range.

1.6.2.1 Performing lung clearance index

LCI is derived from multiple breath washout (MBW) during tidal breathing of either an inert tracer gas (being neither absorbed nor secreted by the body) or by displacement of residual airway nitrogen by 100% oxygen [64]. During the time course of this thesis, the Imperial College/ Royal Brompton Hospital group used a tracer gas technique (using 0.2% sulphur hexafluoride (SF_6) in air), which will be the method described and referred to throughout this thesis.

LCI measurement involves the subject breathing the SF_6 tracer gas through a closed circuit with the inspired/expired gas concentrations being measured by a photoacoustic gas analyser (Innocor® (Innovision, Glamsbjerg, DK)). The subject inspires the SF_6 until a steady state at peak concentration (C_{Init}) has been achieved ('wash in phase'). The gas is then disconnected allowing the subject to breathe tidally on room air, eliminating the tracer gas until $^1/_{40}{}^{th}$ of the original concentration has been achieved (C_{END}) ('wash out phase') – this value relates to the limits of detection of early nitrogen analysers.

If the airways are more narrowed (e.g. from inflammation, mucus retention) or have more structural wall damage, gas mixing is less homogeneous and a greater number of breaths will be required to eliminate the tracer gas and the washout phase will take longer [64].

1.6.2.2 Calculating lung clearance index

LCI is a defined as the total lung turnovers required to eliminate the SF_6 tracer gas (this value hence excluding physiological dead space) adjusted for FRC [64].

LCI is calculated by the cumulative expired volume (CEV) required to reduce the tracer gas to $^1/_{40}{}^{th}$ of its starting concentration, divided by the functional residual capacity (FRC). Through multiple washouts of tracer gas, the FRC can be calculated using the equation:

$$FRC = V_{[TRACER]} / (C_{Init} - C_{END})$$

($V_{[TRACER]}$ = volume of tracer gas exhaled up to end of washout, C_{Init} = starting end-tidal fraction of tracer gas and C_{END} = the final fraction of tracer gas [64]) Figure 1.5 shows an example of a MBW trace.

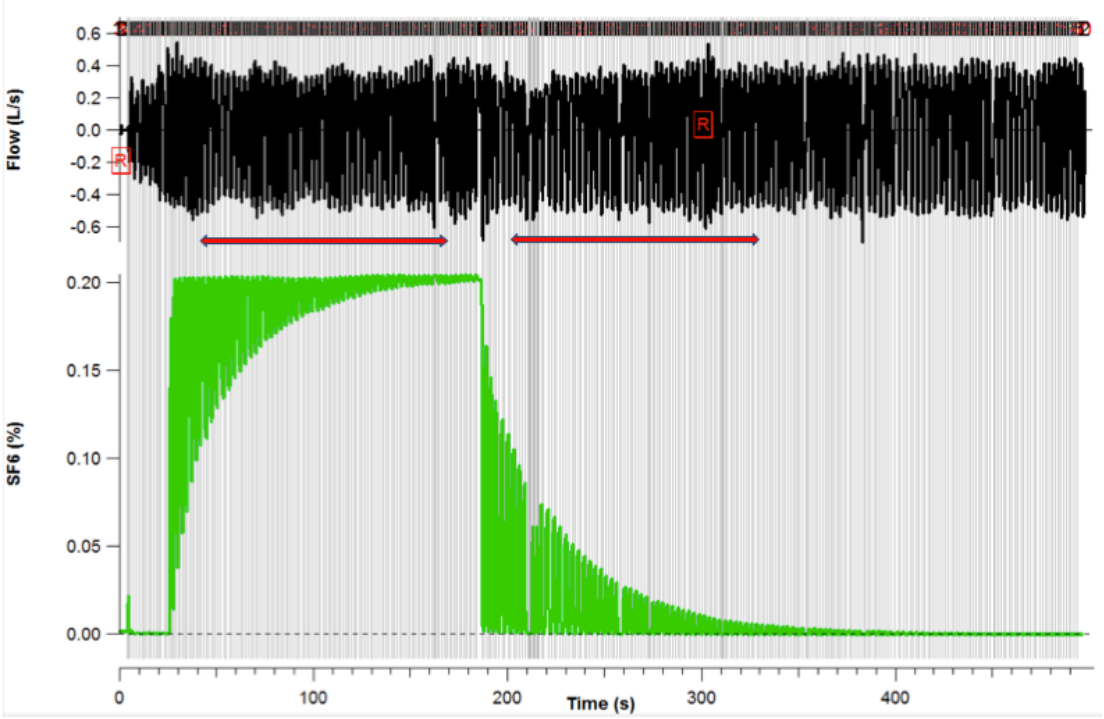


Figure 1.5. Example of a MBW trace to measure LCI. Wash in of the tracer gas, SF_6 (in green) is undertaken until equilibrium (middle) after which the gas supply is disconnected and concentration falls with each subsequent breath as washout occurs (bottom). The black trace denotes flow. (Image courtesy of Katie Bayfield)

Tests are performed in triplicate and are accepted if the calculated functional residual capacity FRC differs between tests <10%. Higher values of LCI would be representative of worse lung disease, however reference values vary depending on several factors including the age of the patient, the MBW device and tracer gas used [65].

LCI has been repeatedly validated to discriminate between patients with CF compared to healthy controls, and degrees of lung disease within CF cohorts. LCI has been demonstrated in numerous CF studies (in children and adults) to correlate significantly with FEV_1, once the latter is reduced. At earlier stages of lung disease, normal FEV_1 can be obtained in patients with an already increased LCI. Additionally, parameters from computerised tomography (CT) of the lungs [66], and other markers of disease severity (e.g. inflammatory markers from BAL fluid and airway infection [67]) have been shown to correlate with abnormal LCI.

1.6.2.3 LCI as a clinical trial outcome

The importance of LCI as a clinical trial outcome is increasingly recognised, particularly where patients have mild lung disease at baseline or demonstrate marginal declines in FEV_1 over time [65]. LCI demonstrated a significant treatment effect in response to ivacaftor in patients (≥6 yr) with a *CFTR-G551D* mutation and mild lung disease (FEV_1 >90%), whilst conventional spirometry (FEV_1) demonstrated much less power [68]; other studies with agents altering sputum rheology (Pulmozyme® [69] and hypertonic (7%) saline [70]) have used LCI to demonstrate a significant treatment effect in children with CF.

1.6.3 Measurement of transepithelial potential difference

In vivo measurements of ion flux across airway epithelial membranes allow a direct and dynamic assessment of CFTR ion channel function, with assessment being possible from the nasal and the lower airway mucosa, with the former commonly used as a surrogate for the latter. In the most simplistic terms a flexible catheter, attached to a recording electrode, is placed into the nasal cavity or lower airway using a technique developed by Knowles *et al* [71] and Alton *et al* [72], and potential difference (PD) measurements made in response to various concentrations of electrolyte solutions.

Ion movement across a cell membrane and the resulting inequality in ionic charge create a measurable PD across that membrane. The characteristics inherent to the CF epithelium of reduced or absent CFTR-mediated chloride efflux, together with the hyperabsorption of sodium, produce a pattern of electrophysiological abnormality distinguishes it from that of a non-CF individual (see Figures 3.1-3.4). As negative ions (e.g. Cl^-) exit the epithelial membrane, the mucosal surface becomes more negative (hyperpolarised); conversely the mucosal surface can become more positive (depolarised) in the presence of more positive ions (Na^+) or less negative ions such as Cl^-.

Chapter 3 will discuss in detail the methods used to measure airway PD and the physiological responses recorded in the healthy and CF epithelium.

1.6.4 Computerised tomography to assess structural lung changes

Computerised tomography (CT) scanning is a diagnostic imaging technique using X-rays to create cross-sectional images of the body, and is considered the gold-standard in CF for assessing morphological changes of the airway and lung parenchyma [73]. Two modalities of thoracic CT scanning are used, namely volumetric (or 'spiral') CT, and high-resolution CT (HRCT) taking thin (<2 mm) scanning 'slices' and used for detailed evaluation of the lung parenchyma [74]. The effects of airway wall inflammation can be observed on CT images of the lung, with bronchial wall thickening being regarded as a sign of airway wall inflammation, and bronchiectasis the result of airway wall remodelling [75]. CT imaging provides an assessment of mucus obstruction (or 'plugging') within the larger and smaller airways, and evidence of disease within the smaller airways with inhomogeneous trapped air ('gas trapping') being seen following on expiratory-phase CT images [73]. These radiological features are demonstrated in Figure 1.6. Evidence of complications of CT diseases can also be investigated using CT images, including diagnosis of pneumothorax, and providing evidence for atypical Mycobacterial infections and fungal disease [76].

Whilst the primary role of CT scanning remains diagnostic, it has developed a role as a surrogate outcome in CF trials having shown correlations with measurements of airway function (FEV_1 and LCI) and airway inflammatory markers sampled by BAL [77]. Several studies have reported CT to be more sensitive to early airway changes than lung function [78], and significant associations between CT changes and the rate of pulmonary exacerbations, lasting up to 10 years, in children with CF have been described [79, 80].

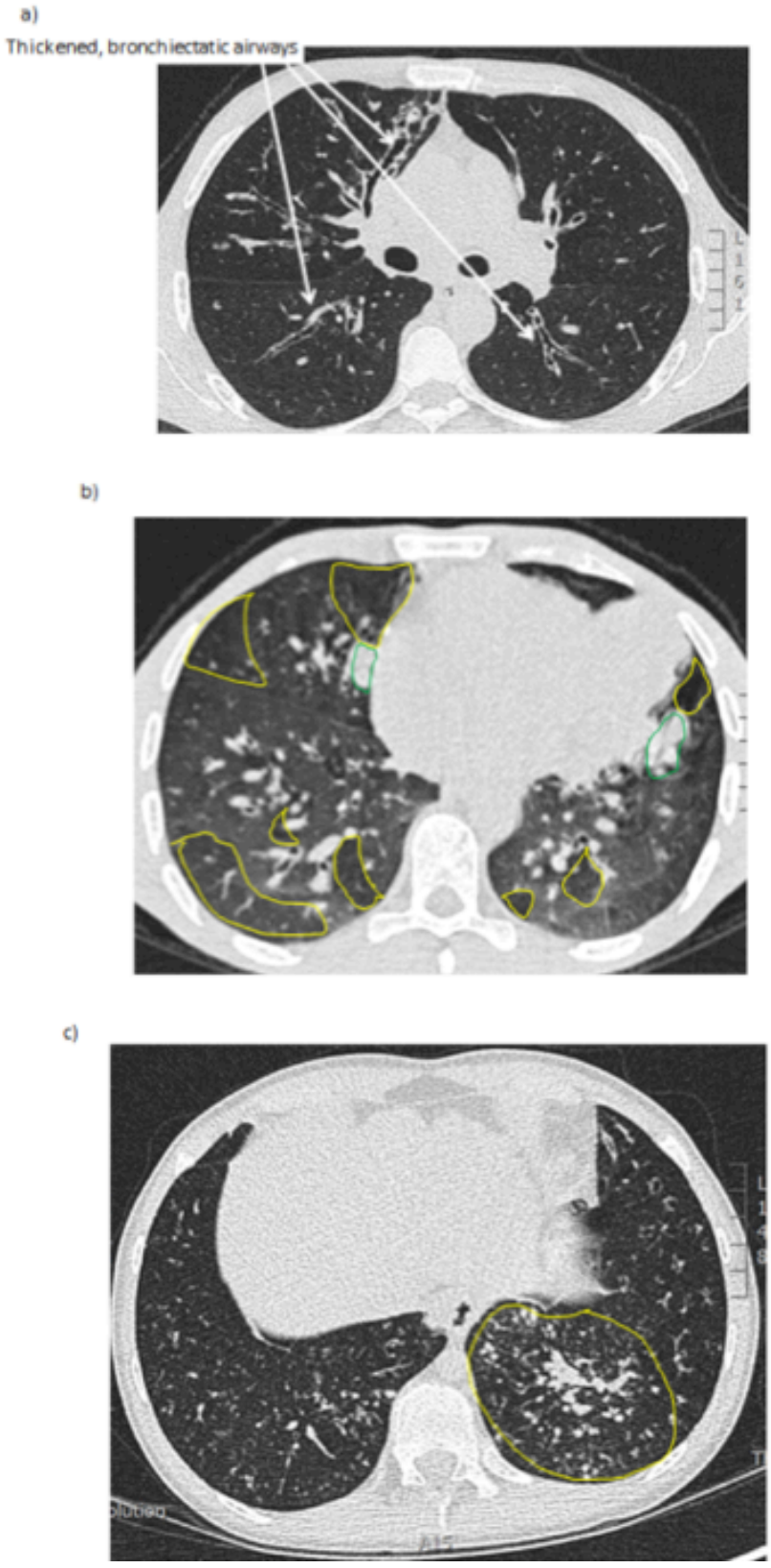


Figure 1.6. Examples of CT images from patients with CF (courtesy of Prof Alton & Davies). Images (top to bottom) (a) bronchiectasis; (b) gas trapping – areas of mosaic attenuation are shown in yellow; (c) mucus plugging within the large airways – highlighted in yellow.

1.7 Diagnosis of Cystic Fibrosis

Diagnosis of CF is made from newborn screening, or from individuals presenting with a constellation of symptoms (Table 1.1), and demonstrating evidence of CFTR dysfunction and *CFTR* disease-causing mutations. The gold standard assay of CFTR function is the sweat test, which uses pilocarpine iontophoresis to stimulate CFTR function in the sweat gland and produce sweat, and the concentration of sweat chloride being measured. In the presence of CFTR dysfunction, elevated levels of sweat chloride are seen. Two further clinical assays of CFTR function exist and are predominately used in cases of diagnostic uncertainty: (i) nasal PD measurement, measuring *in vivo* CFTR function within the nasal epithelium, and (ii) intestinal current measurements (ICM), measuring *ex vivo* CFTR function from rectal mucosa biopsy samples.

1.7.1 Newborn screening for CF

Since 2007, the UK has commenced universal newborn screening for CF by the measurement of immuno-reactive trypsinogen (IRT) in the serum following a Guthrie heal-prick blood spot test [81]. Positive results are next assessed for common *CFTR* mutations. The diagnosis still requires a sweat test for confirmation. The endeavor of newborn screening is to diagnose patients with CF at a pre-symptomatic stage and allow expeditious therapeutic intervention, aiming to slow the rate of decline of lung function, reduce morbidity and increase survival [82].

Prior to newborn screening, 40% of patients were diagnosed in the first 3 months of life and 55% of patients within the first year of life, however since the commencement of newborn screening, almost two-thirds of patients are diagnosed in the first 3 months of life and over 82% by their first birthday. [19]

1.7.2 Diagnosis of 'classic CF'

Most patients present with 'classic' CF disease; in the presence of clinical symptoms suggestive of CF, it is possible to make a diagnosis of CF when the sweat chloride concentration is >60 mmol/L and the patient has 1-2 disease-causing *CFTR* mutations identified from blood sampling [83].

1.7.3 The more difficult diagnosis

The terms 'atypical' or 'variant' CF have been applied previously to patients presenting with at least one organ specific CF symptom and a sweat test with a normal (<30 mmol/L) or borderline (30-60 mmol/L) chloride concentration. A diagnosis of CF is made in this group when patients have 2 confirmed disease-causing *CFTR* mutations; in those with 0-1 mutations, a functional assessment of CFTR is made from either the nasal mucosa by measurement of nasal PD (Chapter 3), or by intestinal current measurements (ICM). ICM measures transintestinal electrical activity on biopsy samples from the rectal mucosa [84]. Intestinal epithelial CFTR is localised at the luminal membrane of enterocytes [85], and in parallel to the airway epithelium the basal, cAMP-dependent and cholinergic chloride secretion of CFTR can be assessed.

A relatively new term 'CFTR-related disorder' is used to describe a clinical entity associated with CFTR dysfunction that does not fulfil the diagnostic criteria for CF despite *CFTR* mutation analysis and functional CFTR testing [86].

1.8 Severity of Disease Phenotype in CF

No matrix exists to clinically define severity in CF, yet it is clear that ranges in severity are seen. Severity is primarily defined by the severity of lung function; the percentage predicted of FEV_1 remains the current best indicator of the

severity of lung disease [87] and prognostic indicator. Original epidemiological data suggested an FEV_1 of less than 30% predicted conveyed a 2 year mortality of over 50% [57]; with the advent of new therapies, patients with an FEV_1 <30% are now demonstrating increased disease stability and marked improvements in survival [88].

CFTR mutations can be a misleading indicator of disease severity correlating poorly with degree of lung disease [89]. Gene modifiers and epigenetic changes, that further enhance or reduce *CFTR* gene expression, are a one explanation for this phenotypic variability [90]; socioeconomic factors and adherence to treatment are major confounders on disease severity and survival [91, 92]. Nonetheless, disease severity is closely associated with the class of *CFTR* mutation, with classes I – III typically presenting a severe phenotype and those with residual CFTR function (classes IV – VI) being milder.

Other clinical parameters are useful in determining severity and extend of disease. Pancreatic exocrine function (pancreatic sufficiency) reflects a better prognosis with patients expressing a milder disease phenotype [93, 94]; improved survival is also seen in patients with lower levels of sweat chloride [95], both features perhaps suggesting residual CFTR function. Frequency of pulmonary exacerbations, adequate nutritional status, and radiological extent of airway disease are also useful clinical indicators of severity.

1.8.1 Relationship between NPD and lung disease severity

Several studies have looked at relationships between nasal PD, respiratory function and genotype; findings are as yet not completely definitive. A correlation between disease severity and NPD has been reported, with patients having more severe FEV_1 impairment demonstrating a higher (more negative) basal NPD and greater response to amiloride, both indices of sodium hyperabsorption [72, 96, 97]; conversely a milder respiratory disease in CF patients was identified with a more normal basal NPD, irrespective of genotype

[96]. A study of adult CF patients (n=79) demonstrated relationships between residual epithelial Cl^- secretion (indicating residual CFTR function) and preserved pancreatic status, and also that a more abnormal Na^+ transport was associated with increased severity of lung disease [98]. A study of older CF patients failed to demonstrate any correlation between survival and residual CFTR function in patients with severe CF mutations [97], yet others have reported that residual Cl^- secretion was associated with a higher FEV_1 regardless of the genotype [99]; a statistical correlation has been reported between stimulated Cl^- transport in male CF patients and FEV_1, however not in a comparable female cohort [100].

1.9 Conventional Treatment for Cystic Fibrosis

When CF was first described in 1938 [101], patients typically died soon after diagnosis; thankfully over the past four decades trends show progressive improvements in survival [102]. With increasing understanding of the condition and with new treatments emerging, life expectancy continues to rise incrementally with a median life expectancy of 40.1 years reported in 2014 (UK) [2].

Specialist multidisciplinary care is now standard in CF, with patient-centred management being coordinated and supervised at designated CF centres [103]. The multidisciplinary team includes doctors, clinical nurse specialists, physiotherapists, dietitians, pharmacists and psychologists with specialist training and knowledge, providing patient care and treatment.

There is currently no treatment universal to all patients that halt the natural progression of the disease; all available therapies merely slow the rate of clinical decline. Existing therapies for CF lung disease may be broadly divided into three categories, each addressing the major complications for disease: (i) clearance of airway mucus, (ii) airway infection, and (iii) optimisation of nutritional status.

These treatments are discussed below, with a summary of the outcome of landmark or major clinical trials.

1.9.1 Airway mucus clearance and reduction in airflow obstruction

To manage the retention of dehydrated, tenacious mucus and airways inflammation, a combination of agents are used with physiotherapy regimes to aid the mechanical movement of airway secretions.

1.9.1.1 rhDNase

The bioengineered product Dornase alfa (Pulmozyme®), a recombinant human deoxyribonuclease (rhDNase), is administered by nebuliser and acts by breaking up extracellular DNA released from neutrophils within sputum, making it thinner and easier to expectorate. Evidence supports it use in CF to significantly improve long-term lung function and reduce pulmonary exacerbations; its short-term use (24 weeks) led to an improvement in FEV_1 of 5.8% with a reduction in risk of exacerbation[104], and improvements in FEV_1 of up to 7% at 12 months with long-term use [105]. It has also been shown to result in reduced rate of progression of airway inflammation [106], and in younger children, improvements in LCI [69]. rhDNase is now a standard of care in CF.

1.9.1.2 Airway surface osmotic agents

Nebulised hypertonic (7%) saline and inhaled dry-powder mannitol both act by creating an osmotic gradient to pull water into the airway lumen, thus hydrating the mucus and ameliorating the defect in MCC. In a randomised controlled trial, improvements in lung function FEV_1 of 3.2% (not significant) were seen after 48-weeks treatment with HTS, as was a statistical reduction in pulmonary exacerbations [107]; further studies have shown statistical improvements in LCI in children treated with HTS [70]. Statistical improvements were reported

following treatment with inhaled mannitol, with an increase in FEV_1 of 7% measured [108].

1.9.1.3 Airway anti-inflammatory agents

To help reduce airway inflammation and airflow obstruction, oral or inhaled corticosteroids (ICS) have long been used. A Cochrane review has concluded that there is however insufficient evidence supporting a benefit of ICS in CF, and owing to systemic side-effects from long-term steroid their use may be harmful [109]. Short-courses of oral corticosteroids are used in clinical practice to treat acute airway inflammation; a Cochrane review of their use long-term (>30 day) identified slower progression of lung disease, however caution regarding their adverse effects was similarly made [110].

The macrolide antibiotic azithromycin is recognised as having immunomodulatory/ anti-inflammatory effects. It has been demonstrated in clinical trials to both increase lung function (the largest being 6.2% [111]) and reduced the frequency of pulmonary exacerbations [112], although the mechanism of action is poorly understood. The use of non-steroidal anti-inflammatory drugs (NSAID) has been studied – the investigators reported that regular use of high-dose ibuprofen (having specific activity against neutrophil chemotaxis) had a reduction in the rate of lung function decline along with reduction in pulmonary exacerbations [113], yet its side-effect profile has limited its use in clinical practice.

1.9.2 Antibiotic therapy

Antibiotics are common practice in CF and can administered to treat acute infective pulmonary exacerbations, to eradicate newly acquired CF airway pathogens, to suppress chronic airway infection or as prophylaxis. They can be administered directly onto the airway surface (nebulised or inhaled), or taken systemically (oral and intravenous antibiotics). Antibiotics that are used for

'maintenance' therapy, aim to suppress or prevent infection, stability in lung function and reduce the frequency of pulmonary exacerbations [114].

1.9.2.1 Antibiotics for a pulmonary exacerbation

Pulmonary exacerbations are treated with short courses of antibiotics, either oral for a mild exacerbation or intravenous (IV) when the exacerbation more severe or complicated; the aim is to reduce the infective load and recover any loss in lung function. IV antibiotics are usually administered as an inpatient in hospital typically for 14 days. This adds a particular burden to the life of the patient, particularly when in employment or education. The ideal situation is to maintain lung health and reduce the frequency of exacerbation, by airway clearance and maintenance inhaled antibiotics.

1.9.2.2 Maintenance inhaled antibiotic therapy

The topical application of regular or cycling antibiotics directly onto the airway surface, either by nebulisation or inhalation, is aimed at suppression of bacterial numbers and predominantly targets chronic airway infection by *Pseudomonas aeruginosa* – 3 main nebulised antibiotics are used in clinical practice: (i) colistimethate sodium (colistin), (ii) tobramycin, and (iii) aztreonam lysine. Recent advances in drug delivery have seen the introduction of dry-powder inhalers for colistin and tobramycin, reducing the time taken for patient administration with a view to increasing treatment compliance.

Despite its use as a first-line nebulised antibiotic, a lack of evidence exists for the clinical benefits of nebulised colistin. In contrast, the aminogylcoside tobramycin has become the 'gold standard' against which new topical antibiotics are benchmarked. It is administered in alternating month on/month off regimen; following 6 months of treatment with nebulised tobramycin an improvement in FEV_1 of approximately 7% was seen, along with a reduction in the risk of hospitalisation for pulmonary exacerbations [115]. Aztreonam nebulised demonstrated superiority against tobramycin in a head-to-head study: after 3

courses taken on alternative months it showed an additional 2.7% improvement in FEV_1, along with a reduction in pulmonary exacerbations [116].

1.9.3 Nutrition

Nutrition and maintenance of body mass are essential in CF, with epidemiological associations being made between good nutritional status and improved lung function and survival [117]. Optimisation of weight by appropriate nutritional, mineral and vitamin supplementation is key, along with the replacement of digestive enzymes and the management of CF-related diabetes (CFRD). There is evidence for a relationship between optimal nutrition/ body mass index (BMI) (defined as the ratio of weight in kilograms to the square of the height in metres) with reduced decline in lung function [118, 119]; impaired glycaemic control has also been associated with poorer lung function and longer durations of pulmonary exacerbations [120], with improvements in lung function seen when insulin therapy is commenced [121].

1.10 Correcting the Underlying CF Defect

Whilst standard therapies target the downstream consequences of disease, correcting the cellular defect and function of the CFTR protein is the ultimate goal. Whilst the obvious treatment target for new therapies are the lungs themselves, the nose is often used as a surrogate in drug development and clinical trials, being more easily accessible for direct drug delivery and less invasive for measurements; whilst it is accepted to be an extension of the lower airway and theoretically to possess similar properties, a definitive comparison has not yet been made of CFTR function in the two sites.

Two separate approaches attempt to restore CFTR function: restoring function of a defective CFTR protein, and inserting a healthy *CFTR* gene into epithelial cells (gene therapy).

1.10.1 Small molecule CFTR modulators

Small molecule modulators, those that improve CFTR channel function by correcting its structure and location ('corrector') or increasing the channel open probability ('potentiator'), are being explored in laboratories and in clinical trials. The molecules are most commonly the results of high-throughput screening technology, a technique that screens batches of small molecule agents against defective CFTR proteins in cells and by *in vitro* measurements of chloride-channel activity, identify possible therapeutic agents [122]. Two such drugs have recently being approved for clinical use, one of which being currently funded by the National Health Service (NHS) for selected patient use within the UK.

1.10.2 Ivacaftor – a novel treatment for *CFTR* gating mutations

Ivacaftor (Kalydeco™), an oral CFTR potentiator, demonstrated significant improvements in CFTR ion-channel function and lung function in CF patients possessing a *G115D-CFTR* mutation (class III 'gating' mutation) on at least one allele [123]. This severe mutation produces pulmonary disease and pancreatic insufficiency and is the most prevalent class III mutation, occurring in approximately 4 to 5% of patients with CF [15]. It results from a substitution within the NBD-1 of glycine for aspartic acid at amino acid 551 [124], and whilst the mutant CFTR protein formed is able to reach the cell epithelial surface, its opening probability is significantly reduced to approximately 100 times less than wild-type CFTR [125]. It is suggested that ivacaftor binds directly to the CFTR protein, enhancing the channel activity and in turn increasing its opening [126].

The phase II clinical trial of ivacaftor used *in vivo* electrophysiological measurements (nasal potential difference) to quantify CFTR ion channel function by chloride-mediated transport [127]. In response to ivacaftor 150mg taken twice daily, the changes after 28-days of treatment were relatively modest (greatest mean change in total chloride response = -5.5 mV) but significant

compared to placebo; the responses returned to baseline after a washout period (7 – 28 days). Parallel changes were seen in sweat chloride concentration, with a significant median change of -59.5 mmol/L in response to treatment, with some patients entering into the non-CF range.

A phase III clinical trial in patients aged 12 years and above reported a dramatic and sustained clinical responses in lung function in response to ivacfator, with an absolute improvement of 10.5% (relative 16.8%) in percent predicted FEV_1 ($ppFEV_1$) being demonstrated at 48-weeks versus placebo. Other major secondary endpoints include reduction of stimulated sweat chloride concentrations, a 55% reduction in risk of pulmonary exacerbations, improvements in BMI and improvement in quality of life questionnaire (respiratory domain); electrophysiological measurements were not made in this study [123]. In a separate study, significant improvements in LCI were reported in children and adults with preserved lung function (mean $ppFEV_1$ >90%) at baseline, demonstrating its benefit in the less severe CF population and the possible sensitivity of LCI as an outcome measure in patients with preserved FEV_1 [68]. This supports that correction of the underlying cellular defect can produce a meaningful clinical improvement.

Since these landmark studies, a further phase III clinical trial (KONNECTION) assessed the safety and efficacy of ivacaftor in other (non-*G551D*) gating mutations, present in around 1-2% of CF patients [128]. It concluded that ivacaftor is safe and efficacious in treatment of this cohort, assessed by clinical outcomes, with absolute increase in $ppFEV_1$ of 10.7% after 8 weeks of treatment, along with improvements in sweat chloride concentrations, BMI and quality of life evaluation; no electrophysiological endpoints were again made. This has led to the pharmaceutical licence of ivacaftor being extended for clinical use in.
The mutation *R117H* (*Arg117His*), present in approximately 4.5% of patients with CF in the UK [2], impairs both the conductance (class IV) and gating (class III) of the CFTR channel [129]. A recent clinical trial of ivacaftor in CF patients carrying an *R117H* mutation demonstrated improvements in $ppFEV_1$ (2.1%, not-significant) and significant reductions in sweat chloride (-24.0 mmol/L);

however subgroup analysis demonstrated that a treatment difference between adults and children, with a significant improvement in $ppFEV_1$ of 5.0% in adults (n=24), and a reduction in $ppFEV_1$ by 6.3% in children (n=9) [130].

Ivacaftor was approved for funding by the National Institute of Health and Care Excellence (NICE) of the UK in 2012 for patients with a *G551D* mutation, and has since been extended to incorporate other (non-*G551D*) gating mutations. Ivacaftor as a treatment for CF patients with the *R117H* mutation has been approved by the U.S. Food and Drug Administration (FDA) in 2014, and by the European Medicines Agency (EMA) at the end of 2015 – at the time of writing, funding has not been approved for its use by NICE.

1.10.3 Ivacaftor in combination with lumacaftor for *Phe508del*

As ivacaftor is a potentiator of epithelised CFTR, it does not work in the commonest *CFTR* mutation, *Phe508del*. Lumacaftor, a CFTR corrector, has been developed to recover function of epithelial cells possessing *F508del-CFTR* mutation, by correcting the conformational folding of the CFTR and allowing successful tracking to the epithelial cell surface. However lumacaftor fails to provide CFTR function in isolation, and requires the addition of ivacaftor. Clinical trials therefore combined lumacaftor with ivacaftor to potentiate remodelled CFTR at the apical surface.

A phase II study enrolled cohorts of *phe508del* homozygous and heterozygous patients to receive monotherapy and combination treatment at differing treatment doses, with the primary outcome being change in sweat chloride concentration [131]. In response to combination therapy, a small yet significant improvement in sweat chloride was measured (-9.1 mmol/L); small improvements in $ppFEV_1$ and quality of life were reported in the treatment groups. No NPD measurements were made in this study.

Two parallel phase III studies (TRAFFIC and TRANSPORT), with near-identical study designs, measured the change in lung function in response to 2 dosing regimens of lumacaftor (400 mg twice daily, or 600 mg once daily) with ivacaftor (250 mg twice daily) [132] compared to placebo. The data reported a modest yet statistically significant improvement of mean change in absolute ppFEV$_1$ from baseline of 2.8% and 3.3% (respective to treatment dose regimen), with pooled analyses demonstrating reductions in number of pulmonary exacerbations and improvements in quality of life (respiratory domain) being reported. No electrophysiological endpoints were assessed in these studies.

Lumacaftor in combination with ivacaftor (Orkambi®) was approved for clinical use by the FDA and EMA in 2015, and is in clinical use in the USA, however its use in clinical practice in the UK awaits a review for approval by NICE (at the time of writing).

1.10.4 Class I mutations and ribosomal read-through agents

Class I mutations account for approximately 5.5% of all *CFTR* mutations in the UK [2]. It was found from *in vitro* studies that the aminoglycoside gentamicin could suppress the premature stop (termination) codons (PTC) found in these mutations and allow ribosomal read-through resulting in a full-length CFTR protein with ion-channel function [133]. Two clinical trials of topical administration of gentamicin to the nasal epithelium of CF patients carrying at least one PTC-*CFTR* mutation, demonstrated electrophysiological (NPD) evidence of *de novo* chloride ion transport though a functional CFTR protein [134, 135]. The adverse safety profile of gentamicin (ototoxicity and nephrotoxicity) however was considered a significant disadvantage and alternatives molecules with a similar mode of action were sought.
In lieu of gentamicin, an orally bioavailable compound, ataluren, was designed to provide ribosomal read-through of PTCs without the unwanted safety profile of gentamicin. In laboratory studies, mice expressing *CFTR-G542X* (a PTC mutation)

demonstrated partial restoration of CFTR channel function in response to subcutaneous injection or oral administration of ataluren [136].

In a phase II study in CF patients with at least one PTC-*CFTR* mutation ataluren was administered orally 3 times per day orally for 12 weeks. NPD demonstrated a mean total chloride response of -5.4 mV compared to baseline. Non-significant improvements in relative $ppFEV_1$ were recorded (4.5%), however a trend was reported between changes in lung function and changes in total chloride response, with both values returning to baseline after ataluren was discontinued [137]. A subsequent phase III efficacy study failed to reach its primary endpoint, failing to demonstrate a significant difference in relative change in $ppFEV_1$ from baseline compared with placebo; neither was a meaningful difference in total chloride response between ataluren treatment and placebo reported, with a similar proportion of subjects in each group showing improvements of greater than -5 mV (ataluren 13% patients; placebo 16% patients) [138]. A post-hoc analysis however demonstrated statistical improvements in the relative change in FEV_1 (5.7%) and reduction in pulmonary exacerbations in patients not taking chronic inhaled tobramycin. It is proposed by the study group that as tobramycin, like gentamicin, belongs to the aminoglycoside drug family, it may interfere with ataluren's mechanism of action at the ribosomal level. This data would suggest that patients with a *CFTR*-PTC but without concomitant use of aminoglycosides might show efficacy from long-term ataluren; a further phase III clinical trial investigating this subgroup has recently commenced (ClinicalTrials.gov identifier: NCT02139306).

1.10.5 Ongoing clinical trials of CFTR modulation

Several novel CFTR modulators are in clinical trials at the time of writing. Two studies are investigating the clinical outcome (FEV_1) in patients with at least one *Phe508del* mutation: (i) phase III multi-centre studies of the corrector (VX-661) in combination with ivacaftor (ClinicalTrials.gov identifier: NCT02516410); (ii) a phase II placebo controlled trial of a new potentiator (QBW251) (ClinicalTrials.gov identifier: NCT02190604). Neither of these studies is measuring epithelial PD as an outcome measure. Two phase I studies are currently active: (i) N91115 is a study medication from a new class of CFTR modulator aiming to increases levels of the cell-signalling molecule S-nitrosoglutathione (GSNO) that is deficient in CF patients, with the aim to increase levels of epithelial CFTR and its function (ClinicalTrials.gov identifier: NCT02275936); (ii) a novel agent, QR-010, an oligonucleotide designed to repair CFTR encoded mRNA using spliceosome-mediated RNA trans-splicing (SMaRT) (ClinicalTrials.gov identifier: NCT02532764). SMaRT is a molecular technology that exploits splicing events during pre-mRNA maturation: by suppressing cis-splicing whilst enhancing trans-splicing, the technology recombines two pre-mRNA molecules, an endogenous RNA target molecule with an RNA trans-splicing molecule (RTM). The engineered RTM delivers wild-type coding regions to replace those of the mutated, endogenous mRNA, thus re-code specific pre-mRNA [139-141]. The niche of this technology would be in genetic diseases, including cystic fibrosis, as an alternative to current models of gene therapy.

1.11 *CFTR* Gene Therapy

Gene therapy is defined as "the introduction or alteration of genetic material within a cell or organism with the intentions of curing or treating a disease" [142]. In contrast to mutation-specific therapies, gene therapy poses an opportunity to ameliorate all *CFTR* mutations by transfer of a "healthy" allele into the cell.

Successful gene therapy requires two key components – a copy of 'normal' *CFTR*-DNA coupled with gene-regulatory components and suitable transfer vector, either biological (viral) or synthetic (non-viral).

At present, *CFTR* gene therapy neither targets stem cells nor incorporates into native DNA. As CF is a lifelong disease and respiratory epithelial cells have a finite life span, any chance therefore of gene therapy providing long-term gene expression and being clinically successful will require repeated application.

1.11.1 UK Cystic Fibrosis Gene Therapy Consortium

The UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC) is a collaboration between Imperial College London, University of Edinburgh and University of Oxford and was formed in 2001 to unify three separate yet similar groups of researchers, each of which had conducted clinical gene therapy trials in CF. The Consortium has been working ever since towards a clinical trial of repeated-doses of *CFTR* gene therapy in patients with CF to attempt to ameliorate the disease, with studies to date having provided proof-of-principle for gene transfer to the airways [143-145].

1.11.2 Pre-clinical *CFTR* gene therapy

Soon after *CFTR* was cloning in 1989, pre-clinical studies demonstrated proof of concept of transfer of healthy *CFTR*-DNA to cells, resulting in gene expression and a functional CFTR protein. Successful *in vivo* transfer of *CFTR*-DNA into pancreatic adenocarcinoma cells (derived from a patient with CF) was established using a retroviral vector; gene expression was reported to produce a protein with cAMP-dependant chloride transport in the CF cell lines [146].

Subsequent animal models demonstrated *in vivo* transfection of human *CFTR*-DNA into airway cells by a recombinant adenovirus vector. Gene expression in

rat airway epithelial cells produced d*e novo* human CFTR protein with stimulated chloride-channel activity, with expression lasting at least 6 weeks following single viral application [147].

1.11.3 Gene transfer in the human airway

To date over 20 trials of *CFTR* gene therapy in man have been published [148]. In order to achieve successful gene transfer, a suitable vector with an optimised gene is necessary.

1.11.3.1 Airway barriers to gene transfer

The airway has evolved, developing physical barriers (mucus and cilia) and immune mechanisms to prevent epithelial damage and penetration by foreign particles and pathogens. Within the CF lung, the increased volume and viscosity of sputum that is laden with inflammatory cells and bacterial DNA further limits the ability of a gene/vector complex to reach the epithelial surface; inhaled particles are trapped in viscous mucus and cleared by cough. Thus, successful gene transfer must overcome these barriers, allowing the gene to enter the cell and, in the majority of current approaches, be able to do so on repeated dosing.

The administration of mucolytic therapies prior to treatment of gene therapy led to an increase in gene transfer by lessening the mucus blockade, however there is at least a theoretical concern over transgene degradation by rhRNase [149]. Pharmacological agents (sodium caprate, perfluorochemical and lysophosphatidylcholine) have been shown in pre-clinical studies to open tight-junctions on the basolateral cell surface and allow viral entry into the cell increasing viral-mediated gene transfer, however the risks of systemic invasion of airway bacteria through these openings likely precludes this approach in clinical practice [150]. Agents to promote ciliary stasis (methylcellulose and carboxymethylcellulose) have been trialled in mice, but never in humans,

increasing respectively viral and non-viral transfer vectors to the airway [151, 152].

1.11.3.2 Amount of CFTR to normalise epithelial function

The vas deferens is exquisitely sensitive to CFTR function; congenital bilateral absence of the vas deferens (CBAVD), present in most male CF patients, can also be an single-organ condition leading to male infertility; these men often have mutations in *CFTR* leading to partial function [153]. Thus, it seems that CFTR function of around 10% of wild-type causes disease in the genital tract but is sufficient to prevent lung disease [150]. This is supported by *in vitro* data, demonstrating CFTR-transfection of 6-10% of CF airway epithelial cells can normalise cAMP-medicated chloride transport [154]. Furthermore, in patients with 'mild' *CFTR* mutations, up to 5% CFTR function appears to prevent (or delay) severe lung disease [155].

Correction of sodium hyperabsorption however requires a substantially greater level of CFTR to restore regulation of ENaC. *In vitro* transfection of human CF epithelium by viral-*CFTR* required close to 100% CFTR to correct Na^+ transport [156], a level unlikely to be achieved with current *in vivo* gene therapy. It is hypothesised that CFTR must directly interact with ENaC to restore epithelial Na^+ transport, therefore this interaction must occur in every transfected cell [156].

However, whilst such levels may be adequate for chloride ion transport, a further increase in CFTR function appears to be required to optimise mucus transport. *In vivo* work has reported that 25% of cultured human ciliated CF epithelia require *CFTR* transfection to improve mucus transport to a level comparable to non-CF epithelium [157].

1.11.4 Viral vectors

Viruses appear the most logical gene transfer agent, having evolved to circumvent airway barriers, invading and surviving within host airway epithelial cells. The suitability of each virus as a vector is determined in part by their ability to bind to airway cells and their capacity to contain the transgene. Viruses utilise cellular receptors on the apical and/or basolateral cell surface to allow endocytosis or membrane fusion, to gain entry into the cell and release their genomic contents [158].

The use of recombinant *Adenovirus* (rAd) and *Adeno-associated* virus (rAAV) to deliver a *CFTR* gene appeared promising in early animal models [159], however clinical trials were less successful. In summary, the studies used nose or lung as the target organ and either rAd or rAAV as the vector. The majority were not placebo controlled, and most were small and single dose (4 studies had repeated application) [148]. The studies assessed the efficacy of gene transfer using NPD as a measure of CFTR function, *CFTR*-mRNA as evidence for gene transfer, or both. Partial correction in chloride transport (as measured by NPD) was found following single virus dosing, but no change in sodium transport; both positive and negative results were seen for *CFTR*-mRNA in the host epithelium. In cases where patients had received repeated viral dosing (rAd and rAAV), a sequential decrease in NPD and mRNA were seen following each subsequent dose [160] suggesting the production of serum antibodies to the viral vector. Studies however reported good safety outcomes, with a few reporting mild, self-limiting 'flu-like illnesses and a transient inflammatory response as a side effect.

To date, there has been only one study of repeated administration of *CFTR* gene therapy that has assessed clinical improvement as an outcome measure. An initial safety and tolerability study of three nebulised doses of rAAV/*CFTR*-cDNA administered to 20 mild CF patients (ppFEV$_1$ 82 ±19%) identified small improvements in lung function, with 5/20 patients demonstrating a 10% improvement 30 days after treatment [161]; however, the subsequent larger phase IIb study demonstrated no clinical improvement in lung function over

time [162]. *In vivo* electrophysiology was not used as an outcome measure in this study.

The underperformance of viral vectors is likely multifactorial. It relates to: (i) a paucity of *Adenovirus* receptors on the apical surface of the lung compared to animal models [158]; (ii) the small size of the rAAV limiting the packing capacity of transgene within the virus; and (iii) neutralising antibodies against both prior exposure to wild-type viral infections, and cellular and humoral immune responses against the vector itself resulting in host immune memory, and rendering subsequent viral-vector administration ineffective [150]. This has led to exploration of other potential vectors.

1.11.4.1 Optimised viral vectors

Currently, pre-clinical studies of a pseudotyped l*entivirus* combined with *Sendai* viral F/HN envelope proteins are in progress within the UKCFGTC. [163]. The *Sendai* virus is highly efficient at infecting airway cells owing to hemagglutinating/neuraminidase (HN) and the fusion (F) glycoproteins on its surface, which allow the virus to bind to cell surface sialic acid and cholesterol residues before releasing genome into the cytoplasm [164]. However, it is not repeatedly administrable and duration of gene expression short (peak expression 48 hr after transfection and returning to baseline within 2 wks) [165]. In contrast, *lentiviruses* such as Simian Immunodeficiency Virus (SIV) can (i) evade the immune system allowing repeated dosing, however the mechanism is unclear, and (ii) integrate into the DNA of the host genome, and persist, with gene expression lasting the life-span of the cell [150]; however, they bind poorly to respiratory epithelial cells. The UKCFGTC has built on the best of both these approaches, by pseudotyping the SIV so that it expresses the F/HN proteins (F/HN-SIV) [163]. Pre-clinical studies to date have demonstrated that the F/HN-SIV (expressing *luciferase*) can be administered to murine lower airways repeatedly without loss of gene expression, and with transduction following only brief contact time (seconds); furthermore, gene expression can last for the life-span of a mouse (approximately 2 years) following a single dose without

evidence of chronic toxicity [163]. *In vitro* studies have demonstrated F/HN-SIV-*CFTR* can transfect fully differentiated human airway epithelium and produce functional cAMP-dependent CFTR chloride channels, however this was not replicated in the CF mouse nasal epithelium – the authors comment that the murine upper airway may be dominated by olfactory rather than respiratory epithelium [166]. A lentivirus (vesicular stomatitis virus G)-*CFTR* complex (i.e. non-F/HN pseudotyped) has been reported to produce 12 months of CFTR expression (as measured by partial correction of chloride transport) following a single dose administered to the upper airway of *CFTR*-knockout mice [167].

The F/HN-SIV vector would therefore seem to be an ideal vector for *CFTR* delivery, yet it is imperative that pre-clinical safety data is robust. Several cases of malignancy have been reported in children with severe combined immunodeficiency (SCID) after a gamma retroviral vector transduced bone marrow, relating to onco-gene activation [168]. In contrast, most lentiviral vectors developed to date are self-inactivating, reducing the risk of an insertion mutagenesis; furthermore the airway epithelium divides slowly in contrast to bone marrow, and is terminally differentiated [169]. Whilst this makes it less likely that mutagenesis will be an issue in the airway, extensive preclinical safety data are required. For this reason, the first wave product of the UKCFGTC was in fact a carefully selected non-viral formulation.

1.11.5 Non-viral vectors

Synthetic vectors, including cations, lipids and compacted DNA nanoparticles, have been developed which have the advantage of an absence of viral partials and thus escaping an immunogenic response, making repeated administration more feasible. Non-viral vectors are predominately internalised via endocytosis into host epithelial cells – the low rate of endocytosis at the apical surface significantly limits efficiency of the synthetics agents as vectors [150].

In total, 5 *CFTR*-cationic lipid vectors (DC-Chol, DOPE, DOTAP, EDMPC and GL67) have been assessed in phase I clinical trials, being administered to both the nose and the lung, with similar outcome measurements being made for efficacy as for the viral vectors.

Successful *CFTR* gene transfer into the nasal mucosa of 15 CF patients was established following a single dose of *CFTR*-liposome (DC-Chol/DOPE). Production of a functional CFTR protein being confirmed using NPD, where 20% restoration of chloride transport up measured compared to non-CF controls (maximal at 3 days post-transfection, but regressing to baseline by day 7) [170], with no change in sodium transport being seen. Assays of *CFTR*-mRNA were either positive or undetectable, even with positive NPD changes [148].
Alton *et al* assessed both the safety and efficacy of a single application of *CFTR*-liposome (GL67DOPE/DMPE-PEG_{5000}) to the nose and lower airway (via nebuliser) of 8 CF patients *versus* placebo (cationic liposome alone) [144], reporting significant improvements in chloride transport in both the nose and lower airway, with approximately 25% restoration in chloride channel being measured in the lungs; no change in sodium transport was again found. The observed correction and gene expression was transient. In addition, the side effect profile was considered sub-optimal for a future repeated application trial. Patients in both active and placebo groups demonstrated a transient yet significant fall in FEV_1 (15%); self-limiting influenza-like symptoms were reported in both treatment groups, those in the placebo group being milder, whilst those in the active group were associated with raised plasma inflammatory markers. It was proposed that an inflammatory response directly against CpG motifs on the transgene may have accounted for the 'flu-like reactions seen in the active group (section 1.11.8.1), with an airway macrophage response to the lipid complex producing minor inflammatory effects in both groups.

Prior to the trial upon which this thesis is bases, only one clinical trial had assessed the efficacy (molecular and electrophysiological) of repeated application of non-viral gene transfer. Hyde *et al* undertook a double-blind,

placebo-controlled trial, nasally administering three doses of *CFTR*-DNA/liposome (DC-Chol/DOPE), each at four-week intervals, to 10 CF patients; 2 patients received a placebo [145]. In keeping with the single-dose studies, CFTR activity, as assessed by chloride transport on NPD, was detected in some patients (some into a non-CF range), although overall results were not statistically significant; again, no change in sodium transport was found. Plasmid-derived mRNA was detected in 44% of nasal epithelial samples taken. No evidence of an immunological response towards *CFTR* was observed throughout the study, suggesting repeated administration without loss of efficacy is possible.

1.11.6 A Summary of viral and non-viral gene therapy trials to date

Reference	Vector	Route(s)	n	Safety concerns	Results
Zabner et al, Cell 1993	Ad	Nose	3	No	• NPD: decreased baseline towards normal values • CFTR mRNA and Protein: not detected
Crystal et al, Nature Genetics 1994	Ad	Nose–lung	4	Yes, transient inflammation at highest dose	• NPD: variable • CFTR mRNA: detectable in nose but not lung
Knowles et al, NEJM 1995	Ad	Nose	12	Local inflammation at highest dose	• NPD: unchanged • CFTR mRNA: detectable mostly at higher doses
Hay et al, Human Gene Therapy 1995	Ad	Nose	9	No	• NPD: partial correction
Zabner et al, J Clin Inv 1996	Ad	Nose	6	No	• NPD: mild normalization
Bellon et al, Human Gene Therapy 1996	Ad	Nose–lung	6	No	• CFTR mRNA and protein : some detected nose > lung
Harvey et al, J Clin Inv 1999	Ad	Lung	14	No	• CFTR mRNA: partially positive but reduced in subsequent administration
Zuckerman et al, Human Gene Therapy 1999	Ad	Lung	11	Flu-like symptoms	• Vector DNA: detectable
Joseph et al, Human Gene Therapy 2001; Perricone et al Human Gene Therapy 2001	Ad	Lung	36	Transient fevers, myalgias	• CFTR mRNA and DNA: Transient detection noted
Wagner et al Laryngoscope 1999	AAV2	Nose	10	No	• NPD: Partial correction • CFTR mRNA: none detected
Aitkin et al, Human Gene Therapy 2001	AAV2	Lung	12	Possible	• mRNA: none detected
Wagener et al, Human Gene Therapy 2002	AAV2	Nose	23	No	• NPD: no change • No change in histology of IL-8
Flotte et al, Human Gene Therapy 2003	AAV2	Nose–Lung	25	Well tolerated, possible side effects reported	• NPD: unchanged
Moss et al, Chest 2004	AAV2	Lung	37	No	• FEV_1: improvement trend • CFTR mRNA: undetectable
Moss et al, Human Gene Therapy 2007	AAV2	Lung	102	No	• FEV_1, sputum markers, antibiotic days: no improvement

Reference	Vector	Route(s)	n	Safety concerns	Results
Caplen et al, Nature Medicine 1995	DC-Chol/DOPE	Nose	15	No	• NPD: partial correction • CFTR mRNA: not detected
Gill et al, Gene Therapy 1997	DC-Chol/DOPE	Nose	12	Possible	• NPD: partial correction
Hyde et al, Gene Therapy 2000	DC-Chol/DOPE	Nose	11	No	• NPD: partial correction • CFTR mRNA: detected in some
Porteous et al, Gene Therapy 1997	DOTAP	Nose	16	No	• NPD: partial correction • CFTR mRNA: detected in some
Noone et al, Molecular Therapy 1999	EDMPC	Nose	11	No	• NPD: unchanged • CFTR mRNA: undetectable
Zabner et al, J Clin Invest 1997	GL67 vs naked pDNA	Nose	12	No	• NPD: significant response with both vectors • CFTR mRNA: non detected
Alton et al, Lancet 1999	GL67	Nose-Lung	16	Flu-like symptoms	• NPD: significant correction • CFTR mRNA: undetectable • CFTR DNA: detectable
Ruiz et al, Human Gene Therapy 2001	GL67	Lung	8	Flu-like symptoms	• CFTR mRNA: partially detectable
Konstan et al, Human Gene Therapy 2004;	DNA Nanoparticles	Nose	12	No	• NPD: partial correction • CFTR DNA: detectable in treatment and some controls

Table 1.2. Summary of the outcomes and safety concerns of gene therapy trials in CF – using viral vectors (top), and non-viral vectors (bottom). Reproduced from Prickett and Jain, 2013 [160].

1.11.7 The optimal vector – cationic liposome GL67A

At the outset of the Wave 1 programme, the optimal gene transfer agent was sought to be moved into a trial to determine clinical benefit. At this time, it appeared unlikely that a viral vector would be repeatedly administrable, so the UKCFGTC focused on non-viral vectors and considered the following: the ability to pass through a nebuliser whilst remaining stable; to be manufacturable in sufficient quantities and meet Good Manufacturing Practice standards; a safe toxicity profile; and able to be administered repeatedly [148].

Pre-clinical studies to evaluate which non-viral vector was most suitable were performed by the GTC in small and large animals, and ultimately a detailed comparative study was undertaken of three synthetic gene transfer agents: 25 kDa-branched polyethyleneimine (PEI), the cationic liposome (GL67A) and compacted DNA nanoparticles. Each was complexed with human *CFTR*-DNA and aerosolised into the airways of sheep. Direct comparisons were made for transgene mRNA expression, and local and systemic toxicity; GL67A was shown to have the highest level of transgene mRNA expression compared to the other agents, with 2/8 treated sheep also having CFTR protein detected. Evidence of mild systemic toxicity (increased temperature, neutrophil count from BAL fluid, and increases in the acute phase response protein, haptoglobin) were reported [171].

Cationic lipid GL67A (Genzyme Corp, Framingham, MA, USA) is a combination of the lipids GL67, DOPE and DMPE-PEG_{5000}, at molar ratios 1:2:0.05, and is made from:

- **GL67:** Cholest-5-en-3-ol (3β)-, 3-[(3-aminopropyl)[4-[(3-aminopropyl) amino] butyl] carbamate]
- **DOPE:** 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine
- **DMPE-PEG5000:** 1,2-Dimyristoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene Glycol 5000)] (Ammonium Salt)

On the basis of these studies, the UKCFGTC has determined that GL67A was the best currently available non-viral vector to take forward into its wave 1 gene therapy clinical trials programme [148].

1.11.8 Optimising *CFTR* gene efficiency

1.11.8.1 Removal of unmethylated CpG dinucleotides

It has been established that unmethylated arrays of cytosine (C) and guanine (G) dinucleotides (CpG) motifs, predominately absent from mammalian DNA but present in bacterial and viral DNA, induce a strong T-cell inflammatory response mediated via a Toll-like receptor, TLR9, and further initiating both innate and adaptive immune responses [172] . As the *CFTR* plasmid used in trials is produced by amplification in bacteria, high levels of these CpGs leading to a proinflammatory response are expected.

It was demonstrated that removal of all CpG sequences from the vector genome would reduce this inflammatory response to control levels [173], but that the retention of even a single CpG motif would still elicit this response [174]. Thus the current *CFTR* plasmid used by the UK CF Gene Therapy Consortium has had all 319 CpG-motifs removed.

1.11.8.2 Incorporation of humanised promoter

For effective transgene expression, the pDNA vector requires both a promoter and an enhancer. First-generation vectors used viral promoters to regulate gene expression, e.g. a cytomegalovirus (CMV) enhancer/promoter, which produced high-levels but short-lived up to 2 weeks transgene expression in both pre-clinical and clinical studies [144]. Replacing a viral promoter with a hybrid human-CMV enhancer and the elongation factor 1-alpha promoter (hCEF1), consisting of both viral and eukaryotic regulatory elements for gene expression, demonstrated an increased duration of transgene expression of up to a month in

pre-clinical trials when aerosolised into the murine lung [174], which has also used in the CpG-free plasmid for the current UK CF gene therapy trials.

1.11.8.3 *CFTR* Plasmid – pGM169

By removal of all CpG motifs and with incorporation of a hCEF1 promoter, the resultant *CFTR*-DNA plasmid, pGM169 (VGXi, Houston, TX, USA), has been specifically designed to provide an increased duration of gene expression along with a reduced likelihood of a post-inflammatory reponse; it is the plasmid taken forward to the clinical trials subsequently described in this thesis (Figure 1.7).

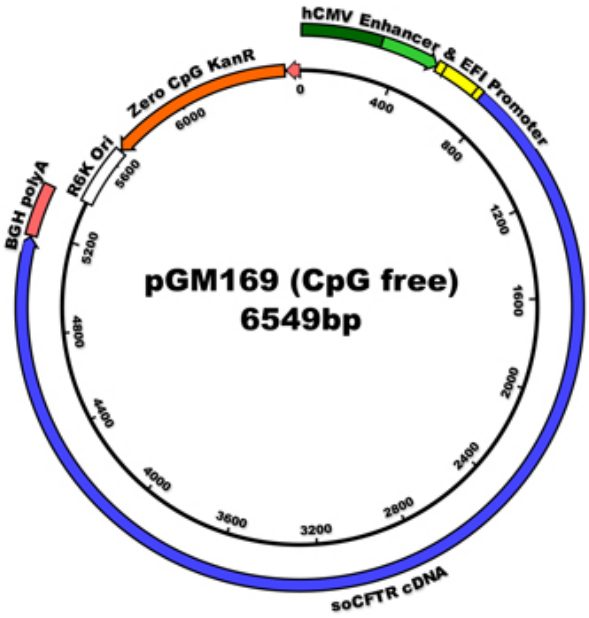


Figure 1.7. Plasmid pGM169. The basic features of pGM169 (proceeding clockwise from 0bp) are: the CpG-free hCEFI enhancer/promoter; a CpG-free synthetic intron sequence to enhance mRNA splicing; a CpG-free version of the *CFTR* coding sequence termed soCFTR2; a CpG-free version of the bovine growth hormone poly-adenylation sequence; a CpG-free version of the R6K bacterial plasmid origin of replication; a CpG-free version of the kanamycin resistance gene, and a CpG-free synthetic bacterial promoter sequence termed EM7. (Image: with permission from the UK CF Gene Therapy Consortium)

1.12 The UK Gene Therapy 'Wave 1' Project

The UKCFGTC established the multi-stage 'Wave 1' project to move pre-clinical work forward, by defining the optimal treatment regime and population to take forward into a clinical trial of repeated-application of cationic liposomal mediated gene therapy (pGM169/GL67A).

'Wave 1' was divided into 3 sequential projects, each rolling into the subsequent clinical phase based on the outcome of the previous trial: (i) the Run-In Study; (ii) the Pilot Study; (iii) the Multidose Trial (chapter 2).

1.13 The Run-In Study

'Longitudinal assessment of clinical measurements in patients with cystic fibrosis in preparation for a clinical trial of CFTR gene therapy' (known as the 'Run-In Study') [175] was a multicentre trial conducted between the Royal Brompton Hospital (RBH), London, and the Western General Hospital (WGH) and Royal Hospital for Sick Children (RHSC), Edinburgh.

The study provided longitudinal data from 192 patients with stable CF (aged >10 yrs; FEV_1 >40% predicted) over a 2-3 year period, with the aims to define (i) the most suitable study population (able to deliver into relatively clean airways, but measurable disease), and (ii) most appropriate endpoints, for a repeated application of gene therapy clinical trial ('Multidose Trial' – Chapter 2. Patients were assessed at 4 time points over the first 12-18 months of the study, then at intervals of approximately every 6 months.

1.13.1 Defining Study Endpoints

The Multidose Trial would require a long enough period of time for measureable clinical changes to occur. Optimal endpoints assays would need to be easily repeatable, reproducible and sensitive to change. The Run-In Study made

measurements of lung function and exercise capacity (spirometry, lung clearance index (LCI) and incremental shuttle walk test), volumetric lung CT scanning, airway deposition and mucociliary clearance scanning, biomarkers of airway inflammation (from exhaled breath condensate and induced sputum) and sputum assays (including viscoelasticity and mucin content). Other selected clinical assays were validated for use as secondary endpoints for the Multidose Trial, those being defined in the Chapter 2 (section 2.5), with those such as exhaled breath content proving of limited use and not included as trial outcome measure.

1.13.2 Defining a study population

The Run-In Study helped define the optimal patient group for the Multidose Trial, those that could permit adequate delivery of gene therapy to the airway epithelium (e.g. less physical barrier to gene transfer from increased mucus and inflammation), whilst being able to measure statistical changes in lung indices (e.g. avoiding patients with supra-normal baseline lung function).

Airway deposition and mucociliary clearance scans were performed by nebulising Technetium-99m labelled human serum albumin at a similar droplet size to the pGM169/GL67A formulation (3-4 μm). As expected, patients with more severe lung disease had more mucus obstruction and increased heterogeneity of deposition, with largely central airways receiving the label. It was concluded that unpredictable, reduced delivery of pGM169/GL67A would be likely in patients with a $ppFEV_1$ below 50%, and for this reason was chosen as the lower threshold of the lung function inclusion criteria for the Multidose Trial.

The study concluded that the best primary endpoint for the Multidose Trial would be the relative change in $ppFEV_1$ after 12, monthly doses of treatment, with data helping define power calculations and study sizes which will be discussed later (Chapter 2, section 2.2.2); patients with a $ppFEV_1$ greater than 90% were considered less likely to demonstrate measureable changes in lung function, so demarcating the upper inclusion threshold.

1.14 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' (the 'Pilot Study') [176] was an open-label safety and efficacy study of a single dose of cationic lipid mediated gene therapy. The aims of the study were to (i) confirm safety following administration of a single dose of gene therapy, then (ii) assess for successful gene expression, (iii) determine a tolerable dose for the repeated application in the Multidose Trial, and (iv) to assess the duration of gene expression following administration into the nose.

1.14.1 Choice of nebuliser

In advance of the Pilot Study, it was imperative to choose a delivery device that was able to aerosolise the gene therapy into droplet sizes suitable for deposition throughout the airways with minimal damage to the pDNA, and have an acceptable delivery rate and efficiency of inhalation. From commercially available CE-marked jet nebuliser devices, an AeroEclipse II Breath Actuated Nebuliser (Trudell Medical International Europe Ltd., Nottingham, UK) was chosen. The AeroEclipse II was able to produce droplets of 3.4 ± 0.1 μm suitable for treatment within the target airway, and is able to operate on a breath-actuated mode, meaning it only delivers particles during inspiration increasing inhalation efficacy. Whilst the AeroEclipse II caused a modest degree of pDNA damage, its was decided by the UKCFGTC that its considerable enhanced airway delivery and lack of inferiority in parameters against other nebulisers made it the most suitable choice for a clinical trial [177]. *In vitro* studies further confirmed that nebulisation onto cell lines did not alter the stability of gene therapy or its ability to produce a functional CFTR.

1.14.2 Pilot Study design and outcome measures

Patients were enrolled to receive a single dose of nebulised gene therapy and/or a dose of gene therapy instilled onto the nasal epithelium. Three nebulised lung doses were used: 5 ml, 10 ml or 20 ml of pGM169/GL67A; based on relative surface area calculations (conducting airways approximately 540 cm^2; nasal epithelium approximately 40 cm^2), a 10% reduction in nasal dose was administered, with patients receiving 1 ml or 2 ml of pGM169/GL67A via nasal instillation. A dose of 10 ml of pGM169/GL67A is equivalent to 26.5 mg of pDNA.

Measurements of safety, and gene transfer and expression were recorded throughout the study. Study visits were made at days 1, 2, 6, 14 and 28 following dosing with gene therapy, with 14 patients receiving a bronchoscopy (20 ml group only) at either day 6 or 14. Safety measurements were made at each visit, and included clinical examination and measurement of oxygen saturations, lung function testing (spirometry, LCI and gas transfer (TL_{CO}), lung CT scanning, and blood sampling (for measurement including the C-reactive protein (CRP), white blood cell count (WCC) and cytokines), and sputum sampling (cytokines). To assess gene expression, transgene mRNA from cell wall brushings were measured, and PD measurements used to assess CFTR protein expression – from either the lower airway (via bronchoscopy) or in the nose (nasal cohort, n=22, at days 2, and days 6, 14, and 28).

1.14.3 Results of the Pilot Study

1.14.3.1 Safety

No major safety concerns resulted from the trial. Two serious adverse events were reported during the study with both being unrelated to the study medication (a recurrence of pancreatitis and a uvula swelling post-intubation for bronchoscopy). A systemic inflammatory response was seen following treatment, with fever, and an increased WCC and CRP (maximal at 8 hours and 2

days respectively) associated with 'flu-like symptoms; often asymptomatic falls in FEV_1 were also seen immediately following dosing, with severity of all these adverse events being dose-related, highest in the 20 ml group and largely minimal in the 5ml group. The adverse events were in general self-limiting and resolved spontaneous within approximately 24 hours. To mask a fever and 'flu-like symptoms, associated with the inflammatory response, and allow blinding within a placebo-controlled (Multidose) trial, anti-inflammatory agents were trialled; pre- and post-treatment with paracetamol successfully abrogated the flu-like response, whilst ibuprofen and prednisolone had no effect. Patients were similarly pre-treated with inhaled salbutamol to reduce any bronchoconstrictive effect of the hypotonic solution. LCI was included in the study as a parameter of safety, demonstrating a statistical increase (worsening) in LCI in the 20 ml group, however statistical decreases (improvements) were reported in both 5 ml and 10 ml groups.

1.14.3.2 Assessment of gene expression

Vector specific mRNA was detected from lower airway endobronchial samples in 2 (20%) patients, with levels being below the limit of quantification in one of these patients; in the nasal brushing samples, mRNA was either not detected (86%) or positive but not quantifiable (14%). This likely reflects the poor sensitivity of the assay, as changes were seen in both nasal and lower airway chloride secretion on potential difference, (i) chloride efflux varied in the nasal epithelium with no overall significant mean change compared to baseline at any time point, however in some patients, chloride efflux was noticed to improve by days 6 and 14, with some patients entering a non-CF range, before returning to baseline; (ii) in the lower airway, 7/10 patients demonstrated improvements in chloride efflux (mean -2.4 mV) following 20 ml of gene therapy. Whilst the PD responses were not significant, the subgroups were underpowered.

1.14.4 Conclusions of the Pilot Study

The Pilot Study concluded that a 20 ml single dose of pGM169/GL67A, whilst tolerated as a single dose, led to side effects which would be undesirable in a repeated application trial. 10 ml was better tolerated, but some patients experienced fever which could unblind a trial, so for this reason, a 5 ml nebulised dose was chosen for repeated application in the Multidose Trial.

The remainder of this thesis is focussed on the Multidose Trial and optimisation experiments that arose during this period.

1.15 Hypotheses and Aims

1.15.1 Hypotheses

The hypotheses for my PhD were:

a) non-viral *CFTR* gene therapy delivered repeatedly would result in correction of basic CFTR function, and lead to improvements in clinically relevant parameters, in particular FEV_1, and reduce the frequent of pulmonary exacerbations;

b) correlations would exist between the electrophysiological changes in ion transport and those in lung function, at (i) baseline, and (ii) in response to gene therapy;

c) electrophysiological measurements in the nose would reflect those in the lower airway i) at baseline, and ii) in degree of change after gene therapy.

1.15.2 Aims

The aims of this PhD were:

a) to complete a clinical trial assessing the clinical responses of patients with CF to repeated treatment with non-viral gene therapy;

b) to make electrophysiological measurements from the upper and lower airway and test for a relationship between measurements of lung function;

c) to understand the relationship of sodium and chloride transport in the CF epithelium between the upper and lower airway by measuring airway PD at baseline, and in response to gene therapy;

d) to validate a novel, 2-operator ('blinded') NPD technique;

e) to understand the importance of amiloride in PD perfusate, and assess for differences in chloride secretion in the nose measured by 2 different PD protocols;

f) to define the minimal interval for performing serial NPDs.

Chapter 2. The *CFTR* Gene Therapy Multidose Trial

2.1 Introduction

This chapter describes the clinical trial 'A randomised, double-blind, placebo-controlled Phase 2B clinical trial of repeated application of gene therapy in patients with cystic fibrosis', which for simplicity will be referred to as the 'Multidose Trial (MDT) throughout this thesis [178].

The MDT is the first clinical trial to assess clinical benefit of repeated non-viral cystic fibrosis transmembrane regulator (*CFTR)* gene therapy (pGM169/GL67A) in patients with cystic fibrosis (CF). Using the data from previous gene therapy studies, and from pre-clinical work and the early Wave 1 clinical trials, this study completed over a decade of work from the UKCFGTC.

The hypothesis of the MDT was that non-viral *CFTR* gene therapy (pGM169/GL67A) delivered repeatedly would result in correction of basic CFTR function, and lead to improvements in clinically relevant parameters, in particular FEV_1, and reduce the frequent of pulmonary exacerbations.

The study commenced in 2012 and was undertaken in its entirety within the time course of this PhD. A large proportion of this thesis is centred on the trial and its outcomes, including those of the electrophysiological response to gene therapy.

This chapter will focus on the methods, conduct and results of the MDT other than those of electrophysiology, which will be discussed in the subsequent chapters.

2.1.1 Author's role on the Multidose Trial

The MDT was conducted during the first 2½ years of the author's PhD, where roles included: writing trial specific standard operating procedures (SOP) and an

adverse events grading table, recruiting and consenting patients, medical care and assessment of patients at trial visits, recording of clinical trial data, administration of gene therapy/placebo to patients, performing nasal PD measurements and nasal epithelial brushing, and assisting with lower airway PD measurements. Throughout the study, the author recorded, graded and assigned causation to adverse events (AE); an audit process was completed to ensure the scoring of AEs between all observers was consistent and equivalent. Following completion of the study, the author's work involved data analysis, primarily of adverse events and pulmonary exacerbations, and of airway PD measurements that were analysed blinded to both trial subject and treatment arm.

2.2 The Aims and Design of the Multidose Trial

The aim of the study was to assess the clinical benefit following repeated doses of non-viral gene therapy administered to the lungs of patients with CF over a period of 48 weeks.

The trial was designed to assess the safety and tolerability of repeated dosing, and to assess gene expression of the formulation over the same period.

2.2.1 Study design

The study design and protocol was developed by the UKCFGTC Strategy Group. This was a double-blind, randomised, placebo-controlled trial, comparing the effects of nebulised treatment with gene therapy to that of placebo (0.9% saline). The study was conducted at three clinical sites – the Royal Brompton Hospital, London (RBH), the Western General Hospital, Edinburgh (WGH) and the Royal Hospital for Sick Children, Edinburgh (RHSC).

It was intended to enrol 130-patients with a mild to moderate severity of CF (as determined by their FEV_1 being between 50-90% predicted) to receive 12-doses nebulised at 4-weekly intervals of pGM169/GL67A or placebo.

2.2.1.1 Choice of placebo

The consortium elected to use (nebulised) 0.9% saline as its placebo, over a *CFTR*-deleted or scrambled plamsid-liposome complex, in part for financial reasons, but also on safety and ethical grounds, choosing to not expose patients to an untested product with potential immunolocigal or deleterious biological responses to an active peptide or novel non-coding RNA molecule. By choosing 0.9% saline, the consortium could further compare treatment with gene therapy against the natural decline in the CF cohort.

2.2.2 Study population size and treatment effect

The primary outcome of the trial was the relative change in percent-predicted FEV_1 after 12 doses from baseline. The study was powered to measure a treatment effect of a relative change on 6% in FEV_1 – this decision was taken by the UKCFGTC Strategy Group (comprising senior clinicians and scientists) and a trial statistician. This was based on other intervention trials in CF that have demonstrated clinical benefit (rhDNase 5.8% [104]; nebulised tobramycin solution 7% [115]; azithromycin 6.2% [111]), and what the Consortium considered was a meaning clinical improvement. The number of patients that could be enrolled was also constrained by the funding available to the study, and related to the cost of material and investigational products.

Based on data from the Run-In Study, it was estimated that a standard deviation (SD) of 10.0% would be seen in the percent change in $ppFEV_1$ over a 12-month study period from baseline and at 12 months (taking the mean of two measurements at both time points to reduce variability). To achieve a 90% power at the 5% significance level (2-sided), to detect a 6% treatment difference

in FEV_1 between placebo and active treatment groups, a sample size of 120 patients would be required; 130 patients were chosen to allow some leeway for patients failing to complete the study.

2.2.3 Funding

The funding for the trial was from the National Institute of Health Research (NIHR)'s Efficacy and Mechanism Evaluation (EME) programme, and the trial sponsor was Imperial College, London.

2.2.4 Ethical and MHRA approval, and protocol amendments

The MDT was approved by GTAC (reference GTAC184) and the MHRA, and registered on the Clinical Trials database (EudraCT reference 2011-004761-33). The study commenced on 6th June 2012 using protocol version 3 (26th March 2012), which was approved by GTAC on 28th May 2012. Modifications to the study protocol, made during the trial period, comprised revision of the timing and duration of patient visits, and accommodation of Patient Identification Centres (PIC) recruitment from other non-study site centres; no change in the study design was made. The appropriate regulatory bodies approved each protocol amendment, with the study being completed on protocol version 6 (21st August 2013).

2.2.5 Study personnel

The Chief Investigator for the study was Professor Eric Alton (EA) (primary supervisor for this PhD thesis). The clinical team in London was led by Professor Jane Davies (JCD) (secondary supervisor for this PhD thesis) and managed by Clare Saunders (CS)*, Clinical trial* Coordinator, and included two Clinical

Research Fellows (the author of this thesis (MDW) and Dr Katharine Harman (KH)), and a team of research nurses and physiologists. The clinical team in Edinburgh was led by Dr Alastair Innes (adult patients, WGH) and Dr Steve Cunningham (paediatric patients, RHSC), and included two Clinical Research Fellows and research nurses at the Wellcome Trust research facility, WGH. The Consortium's site at Oxford University, led by Dr Steve Hyde, designed the plasmid and performed all molecular assays of *CFTR.*

2.2.6 Imperial Clinical Trials Unit

Imperial Clinical Trials Unit (ICTU) was responsible for creating an electronic database (InForm) recording all patients case report forms (CRF) entered from source data and was, in conjunction with the Imperial College Research Office, responsible for the monitoring of the study and quality of source data verification.

2.3 Design and Conduct of the Multidose Trial

2.3.1 Trial design

The trial was designed to randomise 130 patients to receive 12 doses nebulised at intervals of 28 ± 5 days of 5ml of pGM169/GL67A, or placebo (0.9% saline). The UKCFGTC Strategy group, prior to commencing the trial, determined that to be included in the 'per protocol' group a subject must have received a minimum of 9 doses; those missing more than 3 doses were withdrawn from the study. Safety data were analysed as part of the intention to treat (ITT) group.

2.3.2 Dosing subgroups

Two subgroups, within the overall ITT group, were recruited and studied at the London site.

A subgroup of patients (n=24) was recruited to undergo bronchoscopy pre-dosing and upon completion of the study (for the remainder of this thesis this will be referred to as the 'bronchoscopy subgroup').

Another subgroup (n=24) additionally received nasal instillations of 2 ml of the gene therapy product or placebo at dosing visits (for the remainder of this thesis this will be referred to as the 'nasal subgroup').

The intention was that the bronchoscopy and nasal subgroups would include the same 24 patients to allow a comparison of the response to gene therapy between both organs. Patients could volunteer to enter one subgroup, both, or neither, with additional time and investigation constraints often limiting patient choice; additional exclusion criteria were mandated for subgroup inclusion (see section 2.3.5).

2.3.3 'Safety' cohort

An initial intensively-monitored cohort of adult patients (n=20) was studied providing early surveillance for adverse events resulting from repeated dosing. Additional clinical visits and investigations were made 2 days post-dosing for the first three doses, with safety data being presented to a Data and Safety Monitoring Board (DSMB) in advance of full trial recruitment. The data presented to the DSMB identified no safety concerns in this cohort, and received a favourable outcome in September 2012 which authorised the study to continue recruitment. Since no concerns were identified, this group will not be discussed further. The thesis will focus on the overall MDT and study outcomes.

2.3.4 Study sites

Subjects were enrolled and investigated at 3 clinical sites: RBH, London recruiting both adults and children, and WGH and RHSC, recruiting adult and paediatric CF patients respectively. Both sites followed identical protocols and SOPs, and had regular cross-site clinical and strategy meetings.

In all further discussions of the MDT, this thesis will focus on the methods specifically used at the London site where the author was based. However, the results presented will encompass all enrolled subjects on the trial unless specially mentioned.

2.3.5 Inclusion and exclusion criteria

Eligible patients were those that met all inclusion criteria (section 2.3.5.1), in the absence of predefined exclusion criteria (section 2.3.5.2). The inclusion range of a ppFEV$_1$ between 50 to 90% (inclusive) was chosen following the Run-In Study, as discussed in the previous chapter, to allow for adequate deposition of nebulised gene therapy into the airway in the absence of severe lung disease and mucus obstruction (lower threshold 50%), and to demonstrate statistical improvements in lung function in patients with milder and near-normal airways disease (upper threshold 90%).

2.3.5.1 Inclusion criteria

1. Cystic fibrosis confirmed by sweat testing or genetic analysis

2. Males and females aged 12 years and above

3. Forced expiratory volume in the 1st second (FEV_1) between 50% and 90% predicted inclusive (Stanojevic reference equations [62])

4. Clinical stability at screening defined by:
    a. Not on any additional antibiotics (excluding routine, long-term treatments) for the previous 2 weeks;
    b. No increase in symptoms such as change in sputum production/colour, increased wheeze or breathlessness over the previous 2 weeks ;
    c. No change in regular respiratory treatments over the previous 2 weeks

    If any of these applied, entry into the study could be deferred.

5. Prepared to take effective contraceptive precautions for the duration of their participation in the study and for 3 months thereafter (as stated in GTAC guidelines)

6. If taking regular rhDNase (Pulmozyme®) willing, and considered able by independent medical carers, to withhold treatment for 24 hours before and 24 hours after the nebulised gene therapy dose (owing to a concern over potential degradadation of the *CFTR* genomic DNA)

7. Written informed consent obtained

8. Permission to inform GP of participation in study

2.3.5.2 Exclusion criteria

1. Infection with *Burkholderia cepacia* complex organisms, Methicillin resistant *Staphylococcus aureus* (*MRSA*) or *Mycobacterium abscessus* (a

precaution against cross-infection)

2. Acute upper respiratory tract infection within the last 2 weeks (entry could be deferred until clinically stable)

3. Significant comorbidity including:
    a. Moderate/severe CF liver disease (varices or significant, sustained elevation of transaminases: ALT or AST >100 IU/l)
    b. Significant renal impairment (serum creatinine >150 micromol/l)

4. Receiving 2nd line immunosuppressant drugs or intravenous immunoglobulin preparations

5. Current smoker

6. Pregnant or breastfeeding

7. Nasal subgroup only:
    a. Significant nasal pathology including polyps, clinically-significant rhinosinusitis, or recurrent severe epistaxis
    b. Chloride secretory response on nasal PD of > 5 mV

8. Bronchoscopy subgroup only:
    a. Previous spontaneous pneumothorax without pleurodesis
    b. Recurrent severe haemoptysis
    c. Significant coagulopathy

2.3.6 Study enrolment

Patients were recruited by the clinical research teams in London and Edinburgh. In London, eligible patients were identified from a clinical CF patient database at RBH and opportunistically contacted and invited to attend for an introductory

visit. Adult patients in Scotland were recruited in Edinburgh at WGH, while paediatric patients were recruited at RHSC.

In response to a shortfall in recruitment (owing to ineligibility of patients, patients being unable to commit to the time constraints of the trial, or patients already enrolled into other clinical trials), Patient Identification Centres (PIC) were established at several CF centres around the UK to identify prospective patients eligible for the study. Names were forwarded to the London clinical team to complete a screening and recruitment process. Patients identified at the Newcastle upon Tyne CF centre were referred to Edinburgh owing to its close geographical proximity.

Local PIC sites were activated in November 2012, and national PIC recruitment commenced in January 2013, with 16 PIC centres referring patients to an MDT research site during the study. Recruitment closed at the end of June 2013, by which time all eligible patients were enrolled and had commenced dosing.

2.3.7 Randomisation of patients

Following a successful screening visit, patients were randomised by clinical trial staff at each clinical site using the electronic InForm database system, embedded with a randomisation algorithm created by ICTU and Professor Gordon Murray (UKCFGTC senior statistician).

Patients were randomised to receive active treatment or placebo in a ratio of 1:1, stratified for study site, age and ppFEV_1. Randomisation of patients within the nasal and bronchoscopy subgroups was 2:1 in favour of active treatment, to enrich for the actively treated group.

2.3.8 Study visits

2.3.8.1 Consent and screening

Patients were screened for eligibility and consented by MDW or KH. Eligible patients completed an introductory and/or a screening visit including baseline measurement of clinical outcomes before commencing the dosing phase of the trial – clinical parameters measured during the study are discussed in 2.5.

2.3.8.2 Dosing visits

Patients attended for dosing visits every 28 days ± 5 days. At each visit a trial doctor and nurse made a clinical assessment, recorded clinical observations (pulse, blood pressure, oxygen saturations, respiratory rate and body temperature) and performed spirometry. At various pre-determined time points throughout the trial, additional outcome measures were made for either efficacy or safety (section 2.5).

2.3.8.3 Nasal and bronchoscopy subgroup visits

Patients in the nasal subgroup required 3 pre-doing measurements, sometimes necessitating additional visits. Patients in the bronchoscopy subgroup required an additional one-day visit for a pre- and post- dosing bronchoscopy. Each subgroup had PD measurements taken from the relevant organ (nose and/or lower airway) to assess CFTR function at baseline and in response to treatment; epithelial brushing samples were similarly obtained to assess gene expression by measurement of transgene mRNA at the relevant site. Further details of the subgroup visits are discussed in subsequent chapters.

2.3.8.4 Follow-up and exit visits

All patients completing the trial had two follow-up visits, at 14 ± 2 days and 28 ±2 days following their final dose. Patients receiving 9 or more doses were

included in the final data analysis (per protocol population); patients exiting the trial having fewer than 9 doses were offered an optional exit visit, taking predominately safety outcome measurements.

2.3.8.5 Multidose Trial dosing visit flow diagram

The following flow diagram provides an overview of the trial, with a summary of the schedule of the study visits, including those within both study subgroups (Figure 2.1).

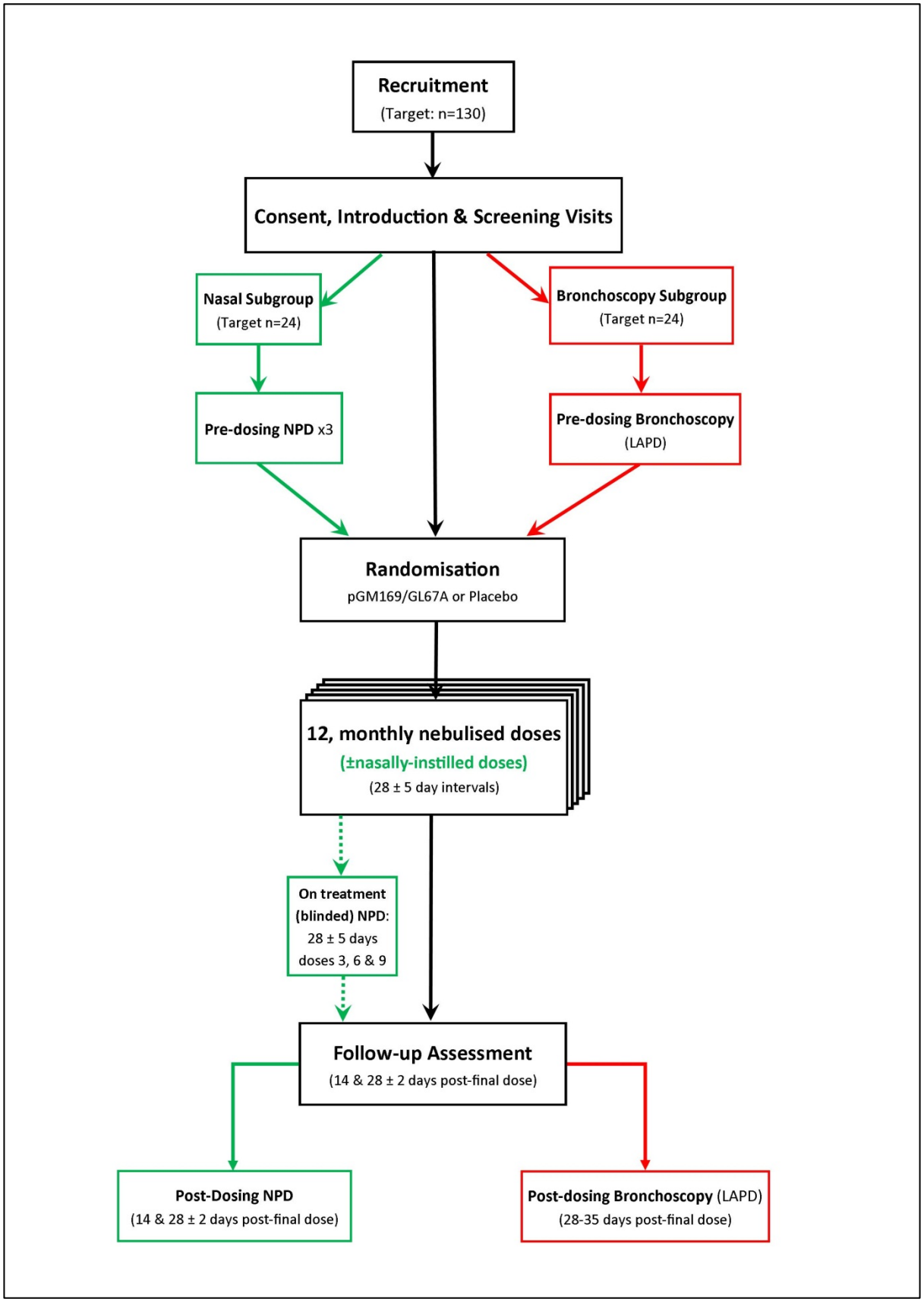


Figure 2.1. Summary diagram outlining the Multidose Trial design and patient visits. GREEN – Nasal Subgroup; RED – Bronchoscopy Subgroup.

2.4 Administration of Gene Therapy (or Placebo)

2.4.1 Preparation of Investigational Medicinal Product/placebo

To adhere to MHRA regulations and to maintain blinding, nebuliser chambers were prepared and filled with either the Investigational Medicinal Product (IMP) or placebo (0.9% saline) by RBH clinical trial pharmacists in a gene therapy formulation room situated in the Royal Marsden Hospital (directly next door to the RBH).

pGM169/GL67A was reconstituted (with sterile water) in 5ml aliquots according to the UKCFGTC SOP. Both components were stored at -80 °C and thawed to room temperature immediately prior to use. GL67A required hydration, and 5.25 ml of sterile water for injection was added before agitation in a vortexer. Rapid mixing of the lipid and DNA was required to form the pGM169/GL67A complex. This was achieved using a purpose-designed, disposable, double-syringe static pneumatic mixer specifically designed by the UKCFGTC for the mixing of lipid with DNA particles.

The IMP was placed into an AeroEclipse II nebuliser chamber, which had been masked in white tape to ensure the contents were not visible to maintain blinding (the IMP is milky white compared to a clear, colourless placebo) and a tamper-proof seal was applied (Figure 2.2); the pot was labelled with the patient's trial details and randomisation code, and supplied to the research team. Alternatively, a placebo (5 ml of 0.9% saline) was added to a nebuliser pot and blinded in an identical manner.

All nebuliser chambers were released from pharmacy at a predetermined hour to prevent the clinical team becoming unblinded by possible delays in reconstitution of the IMP leading to slower preparation than that of the placebo. The nebuliser chambers were transported in designated metallic container to the clinical research facility (CRF), and double-checked by a nurse or doctor against the requisition prescription.

Nasal doses (10 ml of IMP or placebo) were prepared following an identical SOP, and placed in an opaque nasal instillation spray bottle (GSK parts No. AR5989 30mL bottle/AR9488 30mL actuator (GSK, Bedford, U.K.)), similar to commercially available devices for administration of topical nasal decongestants or antihistamines. The nasal device was sealed, labelled, and transported alongside the corresponding nebuliser chamber to the CRF. Whilst 10 ml was prepared, only a pre-calculated volume of 2 ml would be administered to the patient.

The reconstituted products had an expiry time of 4 hours, in keeping with previous stability work within the department (unpublished).

2.4.2 Preparation of dosing nebuliser and nasal instillation device

All administered doses were delivered in a purpose-built gene therapy cubicle in the CRF, with laminar airflow and filtered air extraction. Lung doses were delivered by air-driven nebulisation of the IMP/placebo via an AeroEclipse II Breath Actuated Nebuliser.

The inlet of the nebuliser was connected by plastic nebuliser tubing to a wall-outlet air source, which during dosing would deliver medical air at 6-8 L/min using a breath actuated mode. The outlet of the nebuliser was connected to a PARI mouthpiece and filter set (PARI Medical Ltd, Surrey, UK), as shown in Figure 2.2.

The nasal instillation bottle was primed by spraying 20 times into a blind-ended gauze swab, to prepare the device for product delivery.



Figure 2.2. Photograph representing the set-up of the nebuliser ready for dosing, showing the air inlet tubing, the masked chamber pot, and PARI mouthpiece expiratory filter.

2.4.3 Patient assessment for fitness to dose

A clinical assessment was made by MDW or KH 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 > 40.0%
- Fall in FEV_1 < 20.0% from the last visit (pre-dosing)
- Patient's body temperature (oral) < 37.7°C
- Patient's oxygen saturations (SpO_2) > 94%
- No new abnormality identified at clinical examination
- Not commenced intravenous antibiotics within past 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 dosing being allowed during the study).

2.4.4 Lung and nasal dosing

Dosing of the IMP/placebo was via the breath-actuated nebuliser delivered over a 40 minute period, divided into 8, five-minute cycles of 3 minutes 'on' the nebuliser and 2 minutes 'off'. Patients were instructed to maintain a normal pattern of breathing during nebulisation, and wear a nose-clip to maximise lung delivery and exhalation of any genetically modified into the PARI filter.

Nasal subgroup patients administered 12 doses into each nostril during six of the 'off periods', divided into one spray at the start of the 2 minutes 'off' period, and a further spray into each nostril at the end of the same 'off' period. To administer a nasal dose, patients would tilt their heads forward by 45°, place the nasal spray nozzle into their nostril and lift the bottle to a 45° angle (Figure 2.3), and then whilst slowly breathing in through their noses depress the bottle to generate a spray. All dosing was supervised by a trial nurse or doctor, and the timing of dose delivery accurately adhered to and recorded.



Figure 2.3. Demonstration of the technique to administer the nasal dose, as illustrated by a member of the research team.

2.4.5 Adjuvant dosing medication

To prevent bronchoconstriction, owing to the hypotonicity of the IMP, patients prophylactically received inhaled salbutamol 200-400 mcg delivered via a Volumatic spacer device (Allen & Hanburys Ltd, Uxbridge, UK) 20 minutes pre-dosing; any patient intolerant of salbutamol received no pre-dosing bronchodilator.

To reduce post-dosing systemic inflammatory symptoms (in particular fever) seen in pilot patients, all patients received paracetamol 1 g (or 15-20 mg/kg for children) immediately after dosing and at 6 hours.

2.4.6 Post-Dosing clinical monitoring

All patients remained within the CRF for 2 hours following dosing (doses 1-3), and for 1 hour (later amended to 30 minutes on the recommendation of the author, as no concerning side effects had been observed) for the remainder of doses.

Following a clinical assessment, including measurement of clinical observations, any immediate post-dosing adverse events were documented with patients being discharged by MDW or KH. Spirometry was not undertaken unless there were any clinical concerns, as acute, asymptomatic falls in FEV_1 following active treatment could have led to unblinding.

2.4.7 Risk assessment and infection control

In accordance with infection control policies, all patients were segregated at all times to minimise the possibility of cross-infection, and at the end of every trial visit each room and patient area were cleaned following the hospital SOP. Single-

use equipment was disposed of at the end of the procedure; other equipment was sterilised using Clinell® sanitising wipes (GAMA Healthcare Ltd, UK).

All handling of the IMP was dictated by a trial risk assessment and SOPs. Personal protective equipment (FFP-2 mask, apron and gloves) was worn when preparing the nebuliser and when entering the cubicle within 30 min of dosing; following this period, standard precautions were maintained and a terminal clean of the dosing cubical made. For 30 min following the completion of dosing, patients could choose to remain within the dosing cubical or return to their consultation room wearing an FFP-2 mask to minimise cough aerosolisation of genetically modified particles.

2.4.8 Adverse events

Adverse events (AE) were either patient-reported or clinician-identified (e.g. new abnormal examination findings, new microbiological cultures or deterioration in CT imaging). Patients completed a diary card exercise following each visit, used to recall and help document symptoms and clinical events.

All AEs were recorded by MDW or KH, who assigned causation of the event to its relationship with the IMP study medication (e.g. time-scale in relation to nebuliser dosing, and those reactions expected for the IMP). The severity of AEs was graded using a pre-designated 'Multidose Trial AE grading table' (Appendix A) written by the author and based on published AE tables and guidance [179-181], and local experience.

In the final analysis of AEs, pre-defined overarching groups of adverse event were used, based on clinical features, anatomic systems or biochemical patterns, e.g. increased cough, increased sputum, increased breathless etc. were grouped together as 'lower airway respiratory symptoms'.

To ensure consistency in the recording and scoring of AEs between MDW and KH, and between the two clinical sites, interim AE meetings were held with the clinical leads to review a selection of AEs and an audit process was undertaken which included cross-site review of a proportion of AEs.

2.4.8.1 Serious adverse events

Serious adverse events (SAE) were pre-defined to include any adverse reaction resulting in non-elective hospitalisation (excluding for reasons 'expected' for CF, such as treatment for a pulmonary exacerbation or DIOS) and death. Any SAE occurring during the trial was reported to the Chief Investigator (EA) and the Sponsor of the study (Imperial College) within 24 hours of the event occurring, with mandatory documentation, including the severity and suspected causality, being completed.

2.4.9 Pulmonary exacerbations

The frequency of pulmonary exacerbations (PE) is often used as an outcome measure in clinical trials, yet no standardised criteria exist to define them. Various scoring systems to define a pulmonary exacerbation have been published, using clinical symptoms (change in respiratory and constitutional symptoms), abnormal findings on chest examination, fever, reduction in lung function and changes on a chest X-ray [182, 183]. However, in clinical practice, treatment is often initiated before such protocol-defined criteria are met, which led us to be concerned about under-reporting. For this trial, we therefore elected to collect and report courses of non-elective antibiotics (oral or intravenous (IV)) being administered for pulmonary symptoms. The MDT clinical research fellows did not initiate prescribing for a PE, but at times provided a prescription on the instructions of the patient's own CF clinical team.

2.5 Trial Outcome Measures

Trial outcome measures were recorded at baseline (pre-dosing), throughout the trial (on treatment), and at completion of the study at two follow-up visits or an exit visit (post-dosing). Outcome measures were aimed at assessing clinical efficacy, safety and gene expression. All outcome measurements and assays were collected according to the UKCFGTC trial SOPs at each clinical site. The indices measured during the study are listed in Table 2.1, and a summary of the time each was measured is summarised in Figure 2.4.

Clinical Efficacy	Safety†
Relative change in percentage change in FEV_1*	Clinical examination
Lung clearance index	Transcutaneous oxygen saturation
Quality of Life Questionnaire	Gas transfer
Chest CT scan	Renal and hepatic function
Frequency of additional antibiotics for increased respiratory symptoms	Serum inflammatory markers (CRP, white blood cell count, IL-6)
Relative change in other spirometric measures (inc. FVC, FEV_1/FVC, MEF_{25-75})	Immune response markers (anti-nuclear and double-stranded DNA antibodies, *CFTR*-specific T cell responses)
Change in body weight	Endobronchial histology (bronchoscopy subgroup only)
Exercise capacity	
Activity monitoring	
Serum calprotectin	
Sputum culture	
Sputum weight, cell counts and inflammatory markers	
	Gene Expression
	Potential difference measurements in nose & lower airway
	Transgene mRNA expression in nasal & lower airway brushing samples

Table 2.1. Primary & secondary outcomes for Efficacy and Safety for the Multidose Trial. Indices highlighted in bold will be discussed further within the context of this thesis. (* designates primary outcome; † Safety outcomes include all measures of efficacy)

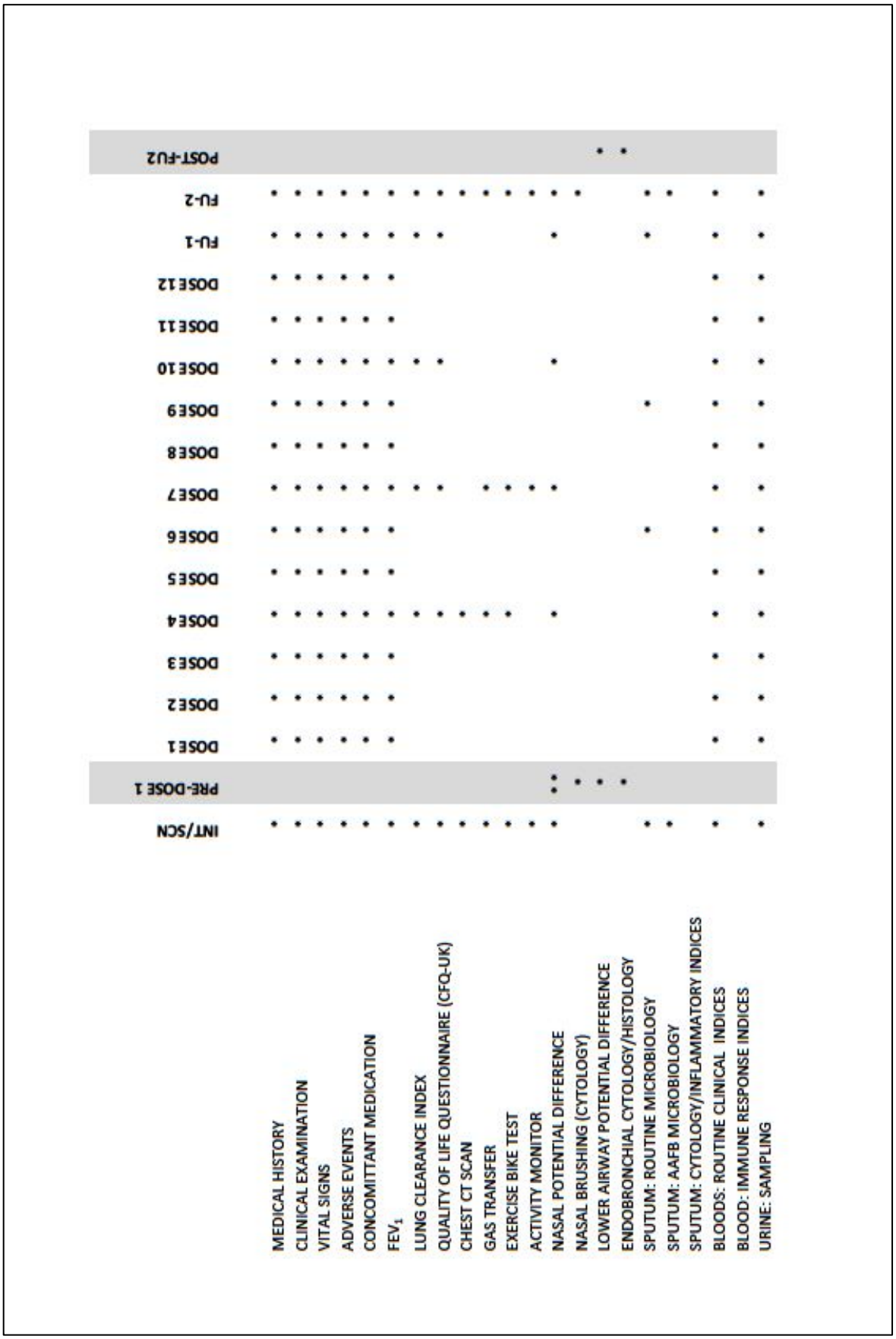


Figure 2.4. Summary of study investigations at individual time points of the Multidose Trial. INT – Introductory Visit; SCN – Screening Visit; FU – Follow-up Visit; AAFB – Acid and Alcohol Fast Bacilli.

2.5.1 Primary endpoint and major secondary outcomes

The primary outcome of the trial was the relative change in percent-predicted FEV_1 after 12 doses from baseline. Major secondary outcomes of clinical efficacy that will be discussed within this thesis were LCI, quality of life questionnaire and lung CT imaging.

A major part of this thesis is focused on the airway electrophysiological responses and transgene mRNA expression – the methods and results of these will be covered separately in chapters 3 to 6, including correlations between the airway electrophysiological responses and changes in the primary and major secondary outcome.

2.5.2 Spirometry

Spirometry was performed at every visit by the trial physiologists in accordance with the current ATS/ERS guidelines [56], using a portable, handheld, battery-operated EasyOne spirometer (NDD, Zurich, Switzerland). The EasyOne was calibrated daily, and prepared for use by entering the patient's height and date of birth onto the digital display, and a single-use EasyOne Spirette (NDD, Zurich, Switzerland) inserted into the spirometer. Patients were seated and wore a disposable nose clip to perform three maximal FVC manoeuvres from a maximal inspiration. The quality and reproducibility of the lung function test was graded by the spirometer, and if necessary was repeated at the discretion of the physiologist and in consultation with a trial doctor.

The best measured FEV_1 was converted from a spirometric volume (in litres) into a percent predicted value based on age, height and gender using a method described by Stanojevic *et al* [184], using an LMSgrowth programme [185] installed on a laptop. This value was used to (1) determine eligibility for enrolment into the study; (2) ensure patients met the fitness to dose criteria (section 2.4.3). The EasyOne also recorded further spirometric measurements,

including FVC, mid-expiratory flow rates and FEV_6 (forced expiratory volume in 6 sec), which will not be discussed in this thesis.

All EasyOne data were saved electronically and downloaded for later analysis.

2.5.3 Lung clearance index

LCI was measured at baseline, at dosing visits 4, 7, 10 and at follow-up. MBW as obtained using a modified Innocor device (Innovision, Odense, Denmark) and 0.2% SF_6 as the tracer gas, following the technique described by Horsley *et al* [186], with all tests being performed by the study physiologists. Strict adherence to infection control measures was observed between patients.

Equipment was set up according to the MDT LCI SOP. The Innocor machine required turning on at least 20 minutes prior to testing to allow the photoacoustic gas analyser to warm up. A closed-loop breathing circuit was assembled (see Figure 2.5): (i) the proximal end was connected to a 0.2% SF_6 cylinder which during testing would be delivered at a rate of 10-15 l/min to prevent re-circulation of gas; (ii) a T-piece in the middle connected the sample port and flow meter to a disposable mouthpiece (separated by a single-use bacterial/viral filter); (iii) an outflow branch of tubing. To minimise ambient SF_6, the door and window of the study room were left open and a fan used to encourage fresh airflow.

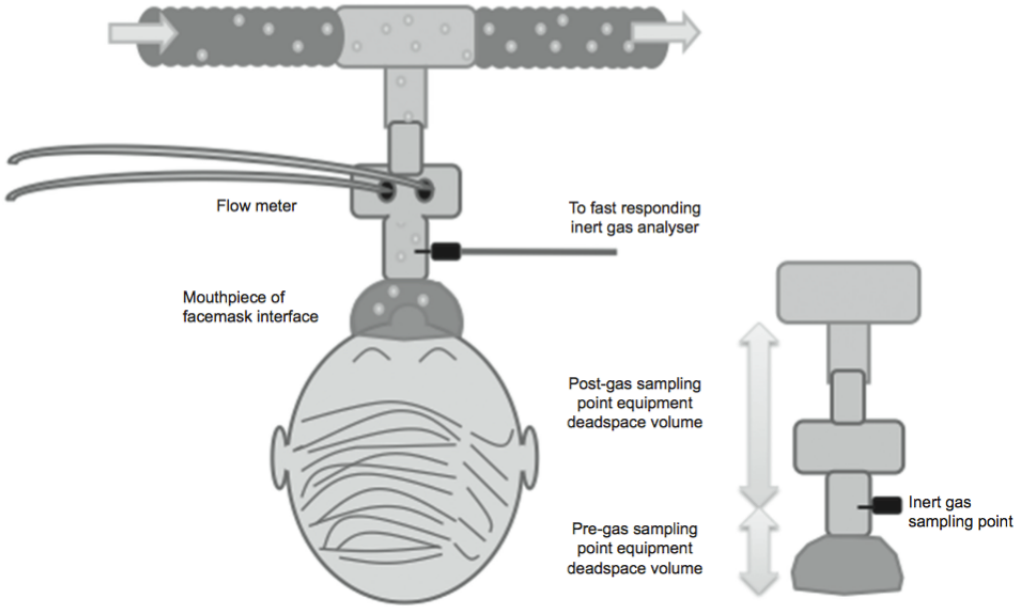


Figure 2.5. Schematic diagram illustrating a generic gas washout system, as used in the multiple breath washout technique for measuring LCI. Reproduced from Robinson *et al* [187].

The Innocor machine was calibrated daily prior to the first test being performed. Subjects would tidally breathe through the mouthpiece whilst wearing a nose clip and inspire the SF_6/air mix until equilibrium with the expired concentration was achieved ('wash in'). SF_6 would be then disconnected and patients would continue to breathe tidally on room air to achieve 'wash out' which was complete once 3 consecutive breaths recorded SF_6 concentrations <2.5% of starting concentration. Three consecutive measurements were made on every subject.

All data were recorded electronically on the Innocor and downloaded for analysis. To ensure consistency in analysis between the clinical sites, every 7th trace was analysed in duplicate by a corresponding team member from the study site.

2.5.4 Lung CT scanning

Computerised Tomography (CT) of the lung was used to assess efficacy of treatment and as a safety outcome. CT scanning was performed by the radiology

departments at each site. RBH patients were scanned on a Siemens Sensation 64 CT scanner (Siemens Healthcare, Camberley, U.K.). Each subject underwent a total of three high resolution CT (HRCT) scans during the study: scans 1 and 3 (pre-dosing and end of trial) were high resolution volumetric scans performed during inspiration from lung apices to below the lung bases (a limited interspaced expiratory phase scan was also performed) and were used to assess treatment efficacy; scan 2 (immediately prior to the 4th dose) was an interspaced (30 mm intervals) HRCT with a maximum of seven sections and was performed for safety reasons. The total effective radiation dose for the entire study was estimated at 4.5 mSv.

CT scans were blinded to treatment arm and phase of scan (pre-/post- dosing), and independently analysed by two pulmonary radiologists. Each scan was assessed using a pre-defined score for a number of indices: the extent and severity of bronchiectasis; bronchial airway wall thickness and the degree of mucus plugging within the large airways and small airways ('tree-in-bud'). For each index, each lobe was assigned a score from 0-4 and a mean calculated; the degree of air trapping within the lung was scored on a percentage basis. Consolidation of lung tissue and ground-glass opacification within the lung parenchyma were used as safety outcomes. A trial administrative assistant entered the final CT data onto InForm.

2.5.5 Quality of life questionnaire

Health-related quality of life (QOL) was assessed using a CF disease-specific questionnaire, the CFQ-UK (Revised) for adolescents and adults (patients 14 year and over) (Quittner, Buu, Watrous and Davis. CFQ-R Teen/Adult, English Version 1.0. Revised 2002; Bryon and Stramik. CFQ-R Teen/Adult, UK-English Language Version 1.0. 2005) [188]. CFQ-R is a reliable and validated instrument used in clinic trials to measure improvements in QOL following an intervention [189, 190], and has been used in trials targeting CFTR function [127, 138].

The tool is a self-administered form assessing 12 domains of quality of life and symptom status. CFQ-R uses a four-point Likert scale with responses for each domain being converted into a composite numerical score between 0 and 100 with higher scores indicating better health. Two domains on the CFQ-R were analysed in the MDT – physical functioning and respiratory symptoms. These outcome measures were not statistically powered, however a minimally clinically important difference (MCID) of 4 points has been established for the respiratory domain [191].

The CFQ-R for adolescents and adults was completed independently by all subjects at the start of specified trial visits; this included patients under the age of 14 to avoid confusion in subjects transitioning from a child to an adult questionnaire during the study and was pre-agreed with the CFQR author, Prof Alexandra Quittner.

All questionnaires were completed on paper and responses converted into numerical values, which in turn were transcribed by the research team into an electronic CFQ-R statistical programme to produce a numerical score for each domain.

2.5.6 Clinical observations

At the start of every trial visit, the patient's height (correct to the nearest millimetre (mm)) and weight (in kilograms (kg), correct to the nearest 100 grams (g)) were recorded; heart rate (HR), blood pressure (BP), respiratory rate (RR) and oxygen saturations (SpO_2) were measured using a Dynamap monitor (GE Healthcare, Little Chalfont, U.K.), and body temperature measured using a single-use oral thermometer (Tempa·DOT, 3M, U.K.) to the nearest 0.1 degree Celsius (°C).

All patients had HR, RR and SpO_2 measured post-dosing; temperature had been noted to increase in response to single-dose IMP (Pilot Study data) and was therefore deliberately omitted to prevent unblinding.

2.5.7 Clinical assessment and examination

Clinical assessment of current symptoms and those occurring between visits was made at every visit by MDW or KH, and any adverse events were recorded. At the start of every visit, a physical examination (auscultation of the lungs, precordium, palpation of the abdomen and bedside assessment of the eyes, ears, nose and throat) was made by MDW or KH. All post-dosing examinations were restricted to chest auscultation, unless otherwise clinically indicated.

2.5.8 Data analysis

All source data were entered onto InForm by the clinical research team, with ICTU monitoring and verifying source data. Two patients had 100% source data verification, with 10% of data verification for the remainder; any queries were flagged and addressed by the research team.

Once data entry was complete, the database was electronically locked and data downloaded as Excel (Microsoft, Redmond, WA, USA) spreadsheets, which were then analysed blinded to treatment arm by the trial statistician and the MDT senior clinicians and scientists.

2.5.9 Statistical analysis

Statistical analyses were performed according to predefined statistical analysis plan devised by the trial statistician and approved by the Trial Steering Committee. An ANCOVA adjustment was used to allow comparisons between

groups – stratification for this was made for the following factors: gender, study site (London or Edinburgh), baseline FEV_1 and whether the patient was in a subgroup. A *post hoc* analysis was performed on all major endpoints, comparing results between high (70-90%) and low (50-70%) starting $ppFEV_1$.

Data was analysed using the statistical software Microsoft Excel (version 14.4.6), IBM SPSS (IBM, Armonk, NY, USA) Statistics (version 22.0) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA)(versions 5c and 6).

2.6 Results

2.6.1 Subject enrolment, withdrawal and completion

2.6.1.1 Screen failure or withdrawal prior to dosing

A total of 191 patients were recruited to the study across both clinical sites. 40 patients failed screening, either because they did not fulfil the inclusion criteria (n=36) or because of clinical instability (n=3). A proportion of these patients had attended for an introductory visit and had completed at least one NPD (n= 6), bronchoscopy (n= 1), or both (n=1) before failing screening. Failure was for the following reasons: identification of a pre-existing or development of a new exclusion criterion (n=5), clinical instability (n=1) or withdrawal of consent (n=2). 15 patients passed screening but withdrew their consent and exited from the study before receiving the first dose of treatment. Patients withdrawing from the study are summarised in a consort diagram (Figure 2.6).

2.6.1.2 Intention to treat population

In total, 136 patients received at least one dose of study treatment and had at least some follow up measurements made; these will be referred to as the 'intention to treat' (ITT) population. In this group, 60 subjects were randomised to placebo treatment and 76 to receive active treatment with gene therapy, the

higher number in the active therapy group reflecting the deliberate randomisation bias of the subgroup participants.

2.6.1.3 Subject withdrawal during the study

20 patients left the study before its completion, either through patient choice (due to lack of time, or commencing the newly licensed drug ivacaftor (patients with a *G551D* mutation)), or from developing pre-defined microbiological exclusion criteria (culturing *MRSA* or *M. abcessus*). One patient exited the study having missed more than 3 doses of treatment, as pre-defined in the protocol.

Whilst a larger proportion of patients withdrew from the study in the active treatment group (n=14, 18.4%) compared to the placebo arm (n=6, 10.0%), this difference was not statistically significant (Fisher's exact test, p=0.22). A further analysis to understand why these patients exited the study concluded that adverse events were not causative and that the reasons for withdrawal did not differ between the two groups (Table 2.2).

2.6.1.4 Per protocol population

The 116 patients (54 placebo, 62 active) who completed the study (received ≥9 treatment doses) are included in the final analysis, and are defined as the 'per protocol' population. The thesis will report data from this cohort, unless specifically mentioned.

A schematic breakdown of the overall patient recruitment, withdrawal and completion is shown in Figure 2.6.

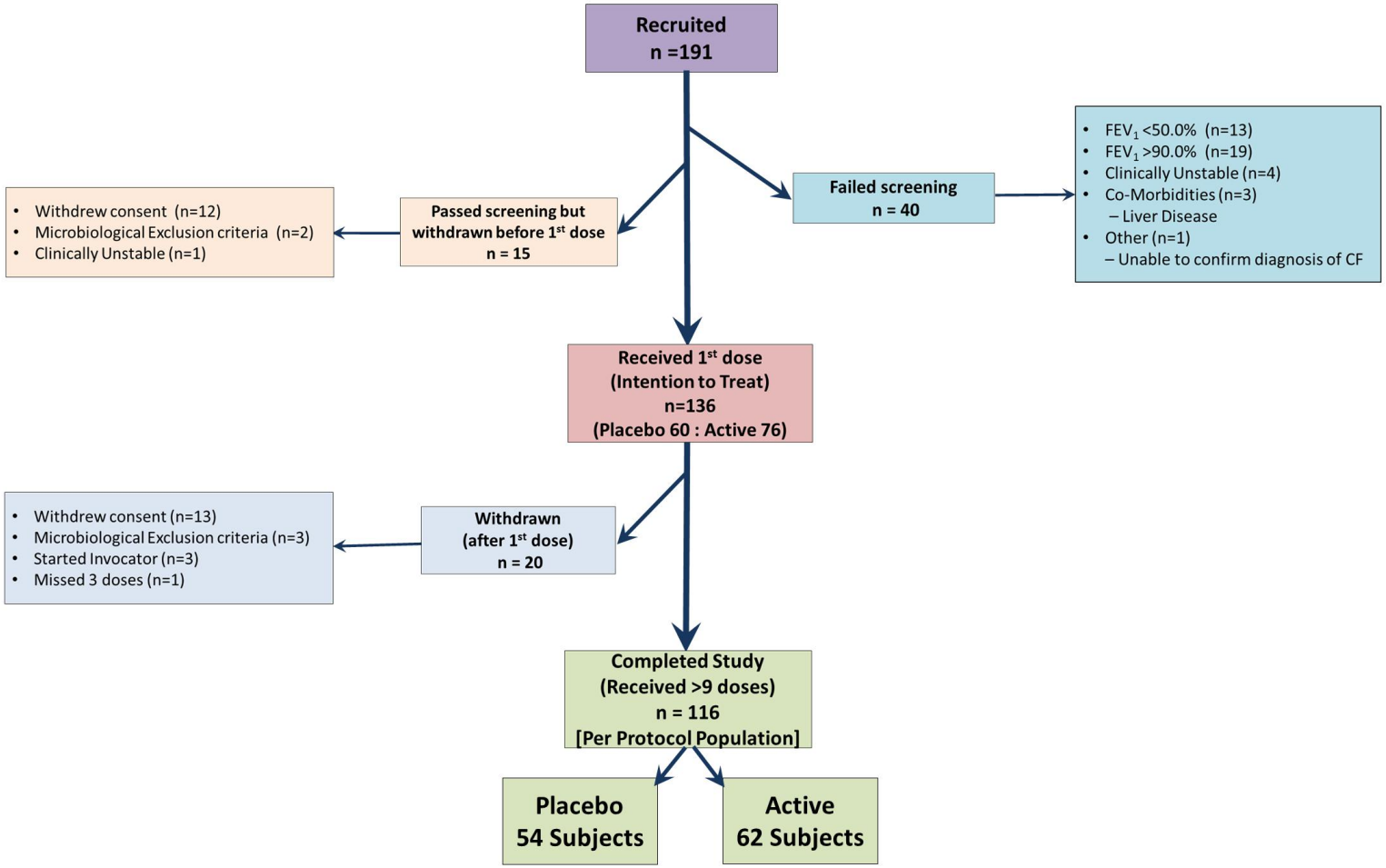


Figure 2.6: Consort diagram reporting patient numbers from enrolment, dosing to completion of study.

Table 2.2. (a) Summary of patients (active group) withdrawing from the MDT having commenced dosing, with reasons for exiting and the number of doses received. SG – subgroup, N – nasal, B – bronchoscopy, WD – withdrawal.

Subject Trial ID	SG	Exit Trial After Dose	Number of Doses (Prior to WD)		Reason for leaving study
			Received	Missed	
LON4006		4	4	0	17-yr old in first year of A-levels. **Withdrew consent - time commitments**
LON4013		9	5	4	Frequent pulmonary exacerbations and low baseline FEV_1 (57%) **Missed >3 doses (6,7,8 & 9)** – not fit to dose all for pulmonary exclusions (low or fall in FEV1 /exacerbation)
LON4043	N	6	6	0	**Cultured *MRSA***
LON4065	B	6	6	0	**Withdrew consent** – elected to discontinue contraceptive precautions
LON4073	B	9	6	3	**Withdrew consent & missed >3 doses** –Missed doses (2,6 & 7) – for GI upset, family holiday and failed to attend dose 10.
LON4074	N	3	2	1	Prolonged inpatient exacerbation for ABPA. Then **cultured *M.abcessus***
LON4105		6	4	2	Missed doses 2 & 6; **withdrew consent – time commitments**
LON4106		6	5	1	Missed dose 2; **withdrew consent – time commitments**
LON4109		1	1	0	**Withdrew consent** after 1st dose – combination of **side-effects** ('flu-like symptoms) and **time/travel issues**
WGH5027		5	5	0	*G551D* – **commenced ivacaftor**
WGH5035		1	1	0	**Cultured *B.cepacia***
WGH5036		3	3	0	*G551D* – **commenced ivacaftor**
WGH5038		2	2	0	**Withdrew consent – new job commitments**
WGH5051		5	5	0	**Withdrew consent**

Subject Trial ID	SG	Exit Trial After Dose	Number of Doses (Prior to WD)		Reason for leaving study
			Received	Missed	
LON4003		7	5	2	Missed doses 3&4 – increased respiratory symptoms. **Patient withdrew consent – increased respiratory AEs** & fatigue post dosing.
LON4053	N	4	4	0	**Withdrew consent – new job commitments**
RHS6003		5	4	1	**Cultured *M.abcessus***
RHS6015		1	1	0	**Withdrew consent – Intolerant of venepuncture**
WGH5013		6	6	0	*G551D* – **commenced ivacaftor**
WGH5048		2	2	0	**Withdrew consent – Time commitments**

Table 2.2. (b) Summary of patients (placebo group) withdrawing from the MDT having commenced dosing, with reasons for exiting and the number of doses received. SG – subgroup, N – nasal, B – bronchoscopy, WD – withdrawal.

2.6.2 Baseline patient characteristics

Baseline patient demographics for the intention to treat group, and those of the 'per protocol' population (receiving 9 or more doses) are summarised in Table 2.3.

	Intention To Treat (n=136)			Per Protocol Population (n=116)	
	Placebo (n=60)	**Active (n=76)**		**Placebo (n=54)**	**Active (n=62)**
Age, yr, Mean (SD)	26.0 (12.8)	25.0 (11.1)		26.0 (13.0)	23.6 (10.8)
<18 yr (n, %)	19 (21.7)	25 (32.9)		17 (31.5)	23 (37.1)
≥18 yr (n, %)	41 (68.3)	51 (67.1)		37 (68.5)	39 (62.9)
Sex, n (%)					
Male	31 (22.8)	38 (27.9)		29 (25.0)	31 (26.7)
Female	29 (21.3)	38 (27.9)		25 (21.6)	31 (26.7)
Study Centre, n (%)					
London	32 (23.5)	49 (36.0)		30 (25.9)	40 (34.5)
Edinburgh	28 (20.6)	27 (19.9)		24 (20.7)	22 (19.0)
% Predicted FEV_1, Mean (SD)	67.9 (10.1)	69.2 (11.4)		69.0 (9.9)	69.9 (11.1)
Body Mass Index, Mean (SD)	22.2 (4.3)	22.3 (4.2)		22.2 (4.4)	22.4 (4.5)
CFTR genotype, n (%)					
F508del/F508del	-	-		26 (22.4)	32 (27.6)
F508del/Other	-	-		25 (21.6)	24 (20.7)
Other/Other	-	-		3 (2.6)	6 (5.2)

Table 2.3. Summary of baseline characteristics from the intention to treat and 'per protocol' groups for both placebo and active treatment arms.

2.6.3 Missed doses

In total, the per protocol population received 696 active doses of gene therapy (mean (SD) 11.3 (1.0) doses per subject) and 609 doses of placebo (mean (SD) 11.2 (1.0) doses per subject); the number of doses missed was 48 and 39 respectively. It is demonstrated in Figure 2.7 that more doses were missed later in the trial compared with the initial period, however this phenomenon was not significantly different between the two groups (Fisher's exact test, p=0.83).

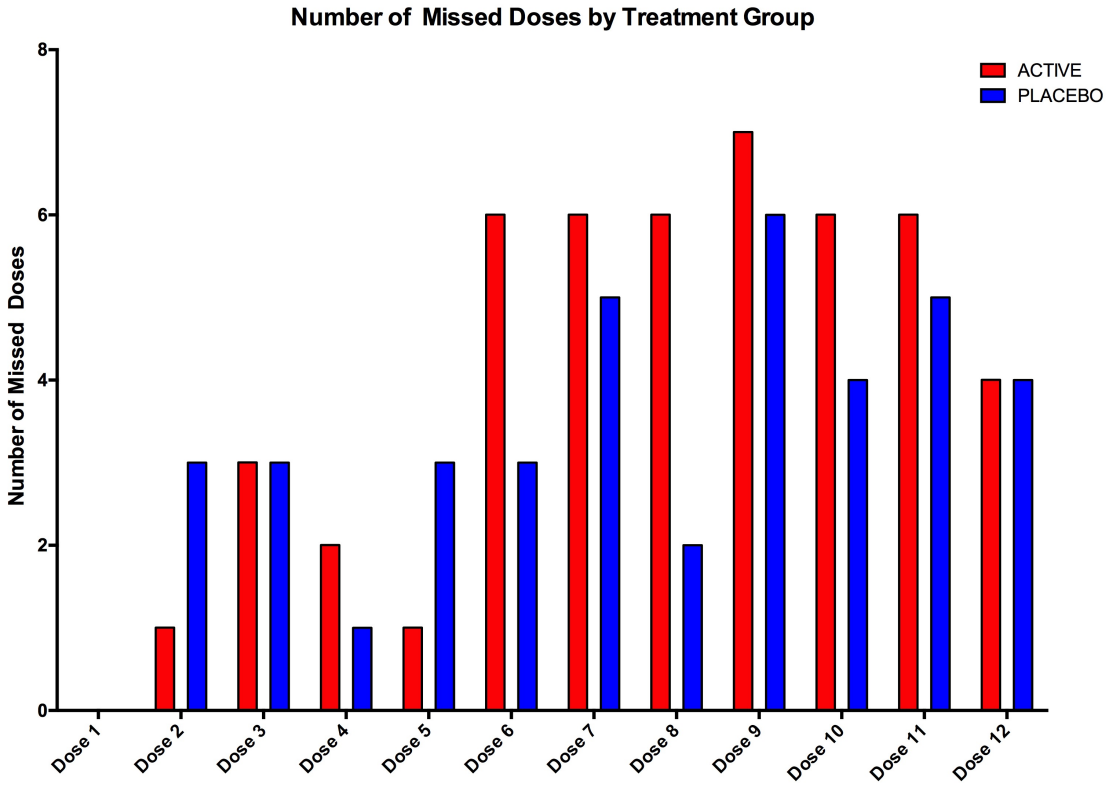


Figure 2.7. Number of patients missing doses per visit per randomisation group

The number of missed doses has been divided into the first half (doses 1-6) and second half (doses 7-12) of the study. In both randomisation groups, there was a significant difference between the number of doses missed in the first compared with the second halves of the trial: active ($p<0.001$) and placebo ($p=0.046$). There was however no difference between the two groups when the division of dosing period is made: doses 1-6 ($p=0.84$) and doses 7-12 ($p=0.59$). Thus a treatment-related effect is unlikely to be the cause.

2.6.3.1 Number of doses missed per patient in the active and placebo group

In total, 29 (46.8%) of the 62 patients in the active group missed at least one dose of gene therapy, and 23 (42.6%) of the 54 patients missed placebo doses. There was no statistical difference between the numbers of doses each patient missed between the two groups; these data are summarised below in Figure 2.8.

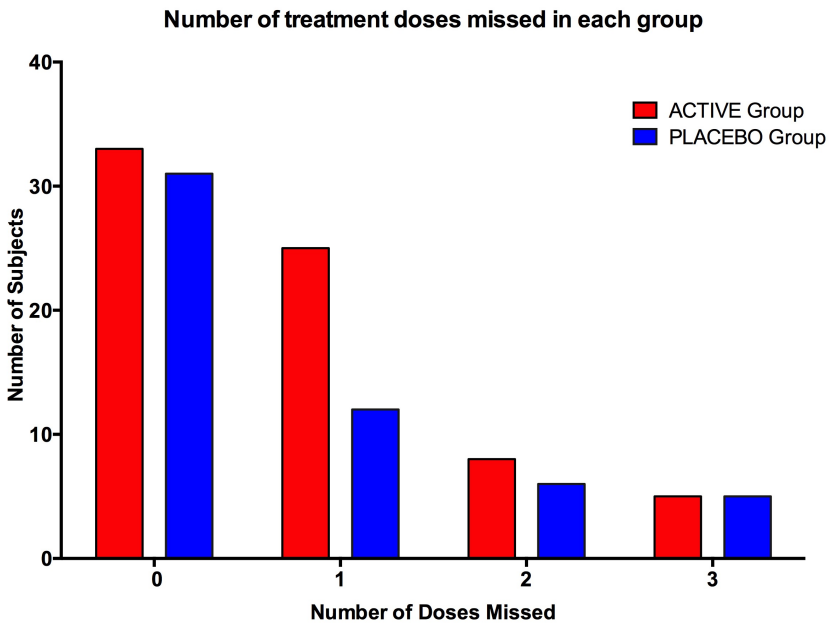


Figure 2.8. Number of subjects from both treatment groups missing up to a maximum of 3 doses of treatment.

In the treatment group, 8 (12.9 %) patients missed at least 2 consecutive doses of gene therapy, however no patient missed both of their final two doses before follow-up; only one subject missed 3 consecutive doses (does 6-8).

2.6.4 Primary outcome: forced expired volume in 1 second (FEV_1)

2.6.4.1 Patients unable to provide follow-up spirometry

Of the 116 patients in the per protocol population, 2 patients (both in the active treatment arm) did not have post-treatment lung function measurements: one

patient experienced an iatrogenic pneumothorax, as a complication from a surgical PORT-A-CATH® insertion, which prohibited forced spirometry; the second withdrew from the study between their final dose and follow-up visit owing to work commitments. Therefore, analysis of the primary outcome was performed on 114 paired lung function measurements.

2.6.4.2 Change in FEV_1 in response to treatment

At baseline, both treatment groups were well matched, with a mean $ppFEV_1$ of 69.0% (placebo) and 69.9% (active). As the trial progressed, the $ppFEV_1$ in the placebo treatment group demonstrated a gradual fall, with a mean reduction of 4.0% at follow-up; lung function in the active treatment group was maintained and demonstrated stability throughout the trial (Figure 2.9). A statistically significant treatment effect was measured between the two groups at follow-up, with a difference between the two groups of in $ppFEV_1$ of 3.7% (p=0.046) (Table 2.4); thus the trial met its primary outcome.

	Placebo (n=54)	Active (n=62*)
Baseline $ppFEV_1$, mean (SD)	69.0 (9.9)	69.9 (11.1)
Follow-up $ppFEV_1$, mean (SD)	66.3 (12.3)	69.8 (11.1)
Treatment Effect†		
Mean Change (95% CI), %	-4.0 (-6.6 to -1.4)	-0.4(-2.8 to 2.1)
Mean treatment effect (95% CI), %	3.7 (0.1 to 7.3) p=0.046	

Table 2.4. Mean (SD) baseline, follow-up (post-dosing) and relative change in $ppFEV_1$ in placebo and active treatment groups (per protocol population). (*Follow-up and change, n=60. †ANCOVA corrected values)

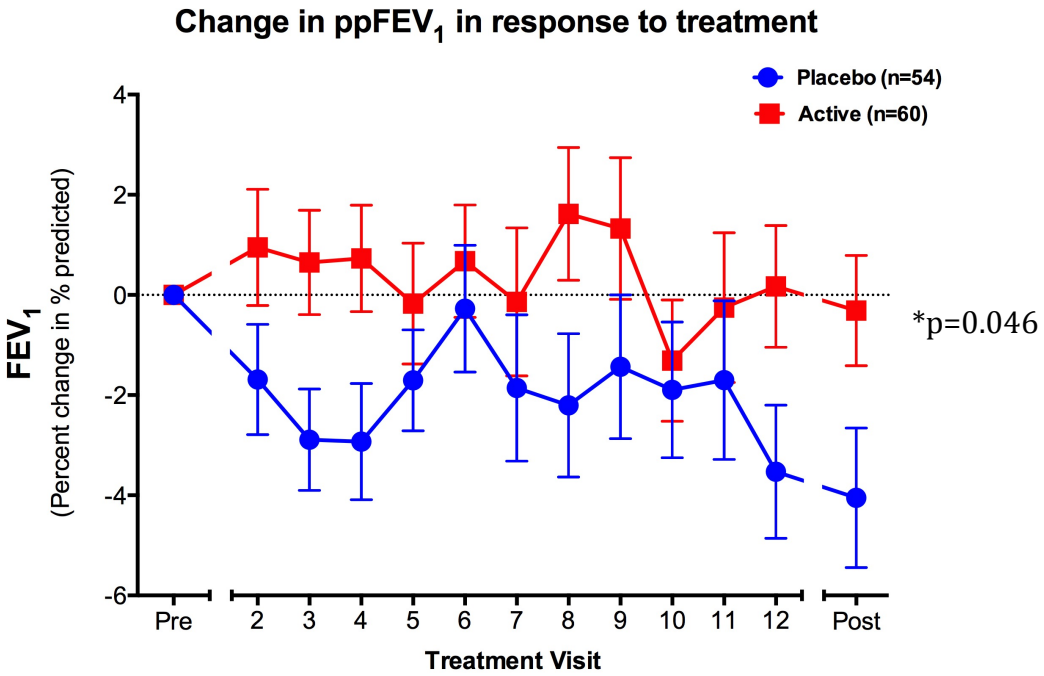


Figure 2.9. Change in mean ppFEV$_1$ in response to treatment with active gene therapy and placebo in the per protocol population (error bars show standard error of the mean). Mean treatment difference 3.7% (p=0.046)

2.6.5 Secondary outcomes: lung clearance index

2.6.5.1 Data analysis and exclusion

LCI measurements were missing from 3 subjects at baseline (2 placebo, 1 active treatment arms), and a further 3 subjects at follow-up (1 placebo, 2 active treatment arms), owing to technical errors within the Innocor system and/or patient choice; in summary, paired data from 51 placebo and 59 active patients were available for analysis.

2.6.5.2 Change in LCI in response to treatment

No statistical change in LCI was detected in either treatment group, with a mean treatment effect of 0.3 (p=0.19) in the active treatment group. The data demonstrate a small deterioration in LCI throughout the study in both treatment

groups, with a suggestion towards a slower deterioration in the active treatment group (Table 2.5 and Figure 2.10).

	Placebo	Active
Baseline		
Number of subjects	52	61
Median (IQR 25, 75)	10.9 (9.6, 12.8)	9.9 (8.5, 11.8)
Follow-up		
Number of subjects	53	60
Median (IQR 25, 75)	11.8 (9.9, 12.9)	10.5 (8.8, 12.0)
Treatment Effect†		
Number of subjects	51	59
Mean Change (95% CI)	0.6 (0.3 to 0.9)	0.3 (0.0 to 0.6)
Absolute treatment effect (95% CI)	0.3 (-0.1 to 0.7) p=0.19	

Table 2.5 Median (IQR 25, 75) at baseline, follow-up (post-dosing) and mean relative change in LCI in placebo and treatment groups (per protocol population). (†ANCOVA corrected values).

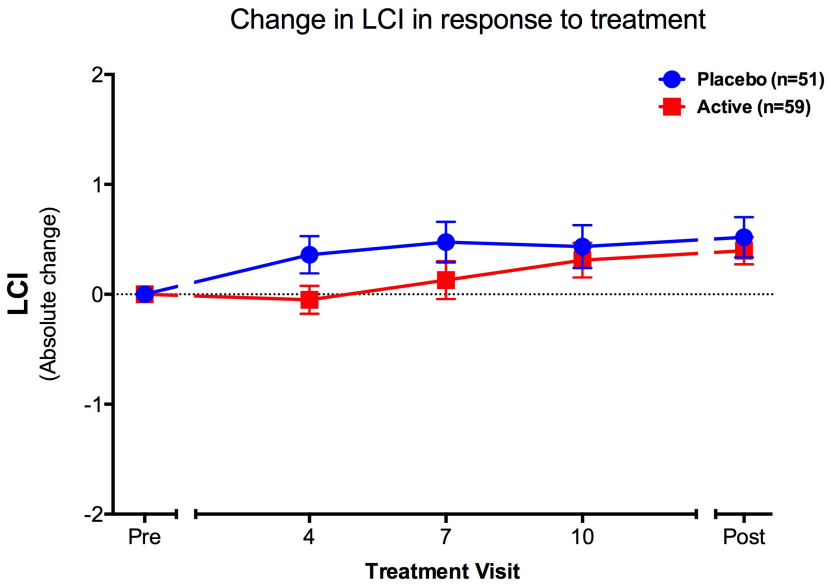


Figure 2.10. Absolute change (SEM) in LCI in response to active and placebo treatment.

2.6.6 Secondary outcomes: CT scan for treatment efficacy

Radiological features of CF lung disease (section 2.5.4) were analysed and assessed for efficacy of treatment. Each radiological index favoured active treatment, with improvements in gas trapping reaching statistical significance ($p=0.048$) and mucus plugging of the small airway being close to significance ($p=0.06$); changes in other CT parameters failed to reach statistical significance. The response of each CT index to treatment is shown below in (Figure 2.11).

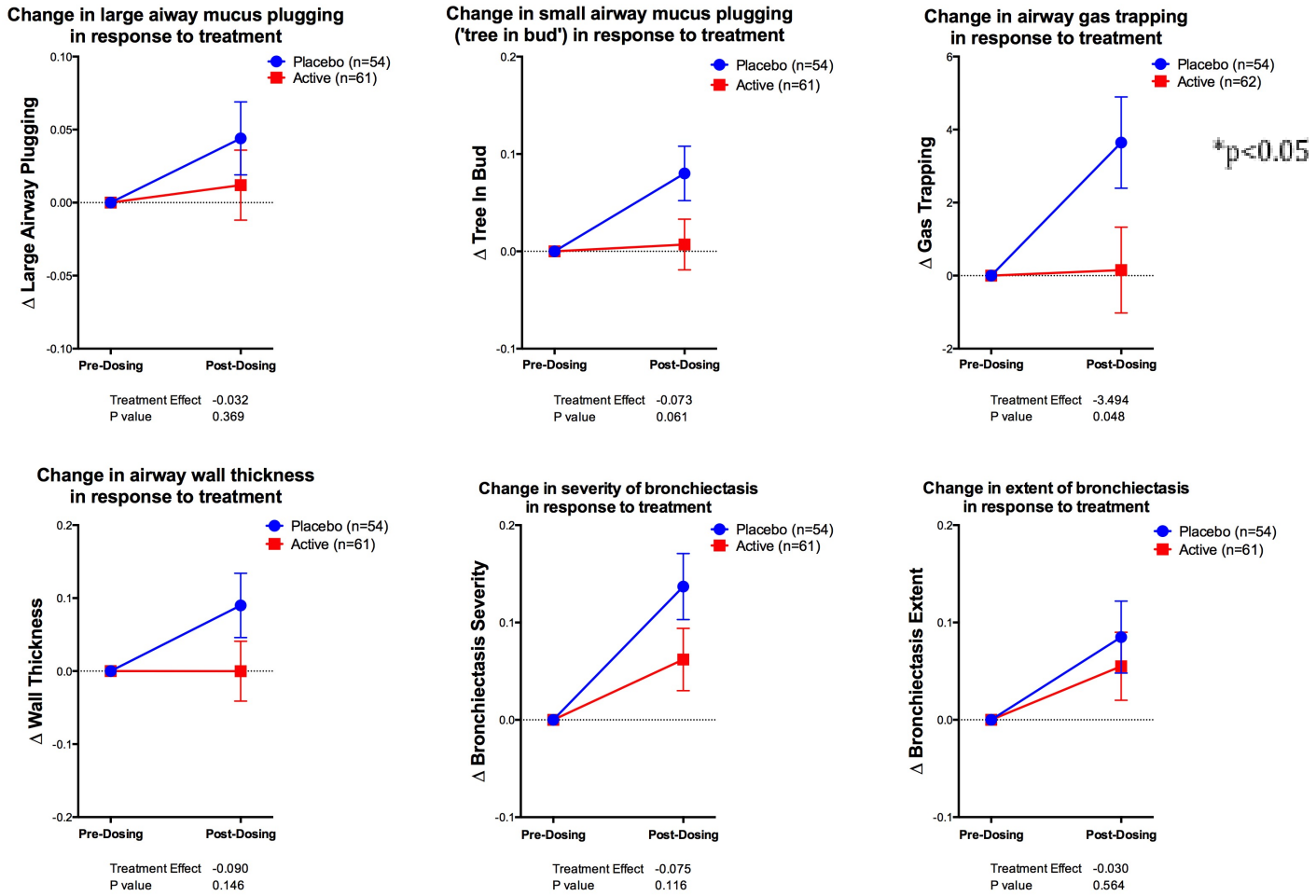
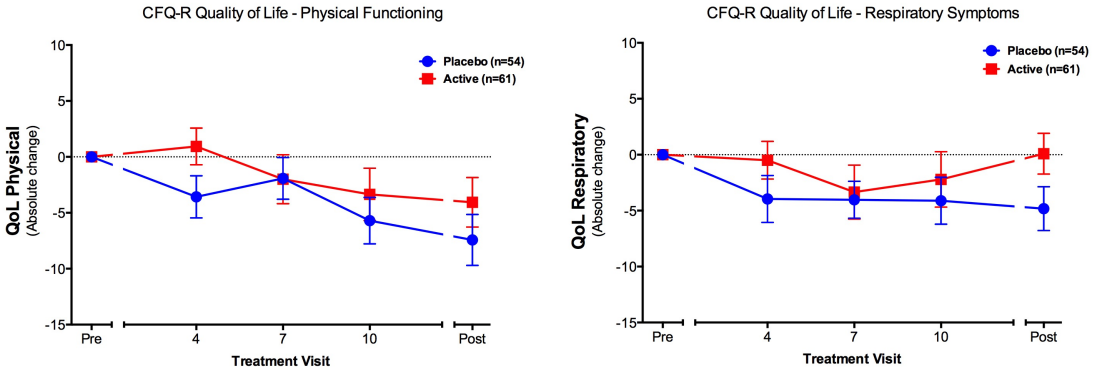


Figure 2.11. Graphs demonstrating changes in CT parameters (individually labelled) in response to placebo and active treatment; corresponding treatment effect and P value (ANCOVA corrected data) are shown. (*p<0.05)

2.6.7 Secondary outcomes: Quality of Life

The physical functioning domain demonstrated a fall in patient assessed QOL in both treatment groups, with a mean (SD) fall following placebo treatment of 7.4 (16.7) and active treatment of -4.1 (17.2); the overall treatment effect (95% CI) was calculated as 1.8 (-4.8 to 8.4, p=0.58). The respiratory symptoms domain demonstrated stability in response to active treatment (mean (SD) = 0.1 (14.3)), compared to a mean (SD) deterioration of 4.8 (14.4) in the placebo arm, with the overall treatment effect (95% CI) of 2.1 (-3.1 to 7.2, p=0.43)) favouring gene therapy. These data is shown graphically in Figure 2.12.



Figures 2.12. Graphs demonstrating change in the CFQ-R QOL parameters in response to placebo and active treatment – (i) LEFT: physical functioning domain; (ii) RIGHT: respiratory symptoms domain.

2.6.8 Summary of primary, main secondary and safety outcome parameters

Outcome measures have been summarised in a forest plot (Figure 2.13), which includes standardised treatment effects to allow comparison of different outcome measures. This figure also includes other parameters and safety indices that were not the focus of thesis, i.e. FVC, MEF_{25-75}, measurement of alveolar

function (KCO_c, $TLCO_c$ and alveolar volume, VA), and haematological indices (CRP, ESR and WBC).

The study was powered to detect a statistically significant difference only in the primary outcome measure, FEV_1. While the study was not powered to detect differences in other outcome measures, a trend was seen across these parameters favouring active treatment over placebo, though they failed to reach statistical significance.

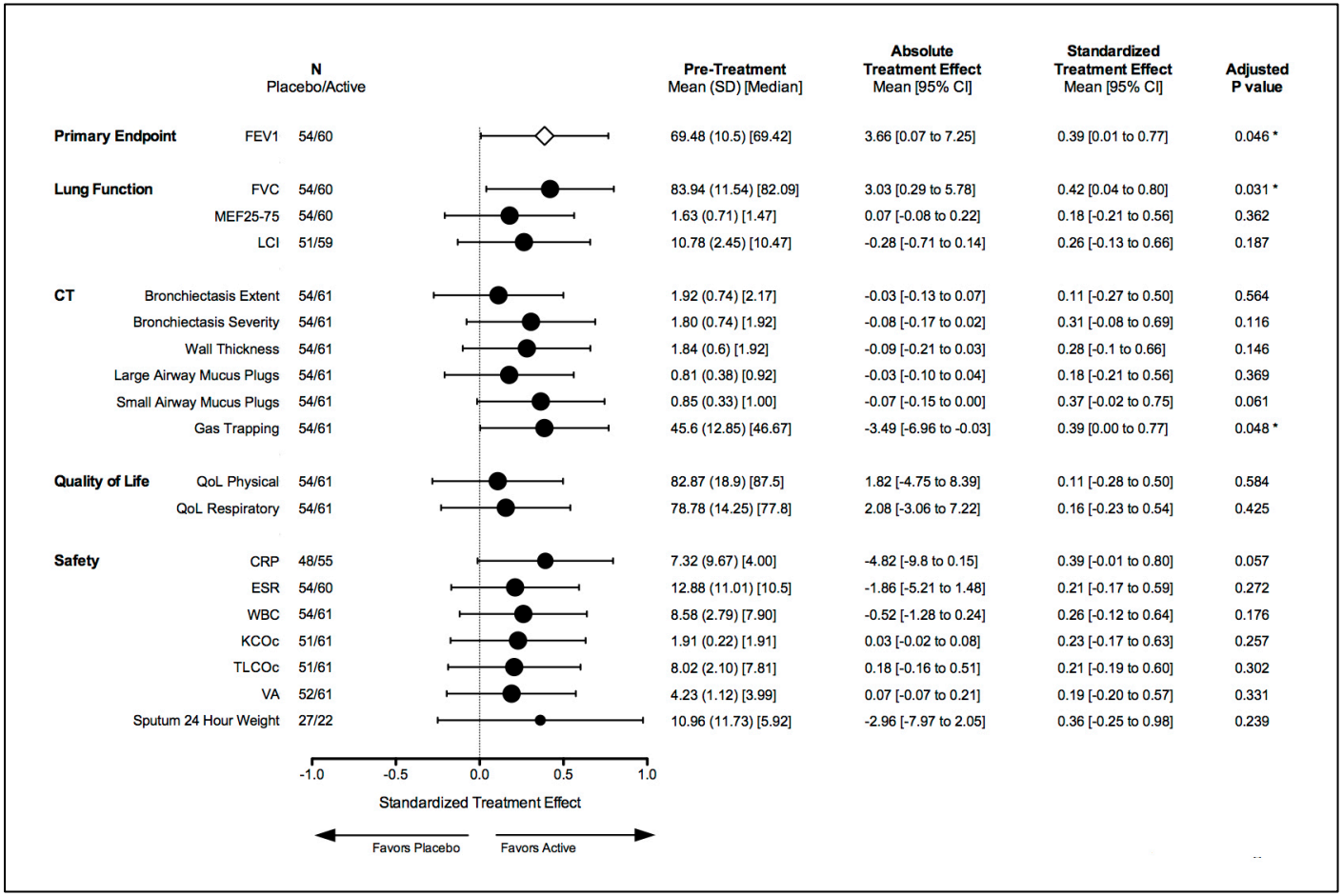


Figure 2.13. Forest plot summarising the primary and secondary trial outcomes, including safety indices, in response to placebo and active treatment. Data shown are the mean (SD) or mean (95% CI) unless otherwise stated. The size of each circle indicates the number of subjects represented; the error bars display the 95% CI.

2.6.9 Treatment effect by baseline demographics

No significant differences in the FEV_1 treatment effect in response to gene therapy were identified when analysed according to age, gender, *CFTR* mutation class (*Phe508del* homozygous versus other), chronic airway infection with *Pseudomonas aeruginosa*, or concomitant CF drugs (data are not presented) [178].

A *post hoc* analysis was performed, stratified by baseline FEV_1, to investigate the effect of severity of airway disease on response to treatment. The per protocol treatment groups was separated into 2 populations: (i) below median baseline FEV_1, ppFEV1 50-70%, and (ii) above median baseline FEV_1, $ppFEV_1$ 70-90%. Analyses of effect of treatment on lung function (FEV_1 and LCI), thoracic CT changes and QOL were made; none was adequately powered owing to the small subject numbers within each group.

Results for each parameter are summarised below.

2.6.9.1 Subgroup analysis – change in FEV_1

In the lower baseline subgroup, the placebo group demonstrated a gradual fall in FEV_1 throughout the study, to a mean (SD) reduction of -5.3% (11.7) whilst the active subgroup demonstrated an increase in FEV_1, which persisted for the duration of the trial: mean (SD) improvement in FEV_1 1.9% (7.9) at follow-up. The combined mean (95% CI) treatment effect was 6.4% (0.8 to 12.1).

No treatment effect was identified in the higher baseline subgroup, with both placebo and active groups demonstrating an almost parallel reduction in FEV_1 (mean (SD) change: placebo = -2.6 % (8.2); active = -2.1% (8.7), with an overall treatment effect (95% CI) 0.2% (-4.6 to 4.9).

The subgroup results and graphical trend of data are summarised in Table 2.6 and Figure 2.14.

	Low Baseline FEV_1 ($ppFEV_1$ 50-70%)		High Baseline FEV_1 ($ppFEV_1$ 70-90%)	
	Placebo (n=29)	Active (n=28)	Placebo (n=25)	Active (n=32)
Baseline Mean (SD), %	61.4 (5.2)	60.0 (5.5)	77.6 (5.9)	79.1 (5.6)
Follow-up Mean (SD), %	58.0 (7.5)	60.8 (7.7)	75.9 (9.6)	77.6 (6.7)
Treatment Effect†				
Mean Change (SD), %	-5.3 (11.7)	1.9 (7.9)	-2.6 (8.2)	-2.1 (8.7)
Absolute treatment Effect (95% CI), %	6.4 (0.8 to 12.1)		0.2 (-4.6 to 4.9)	

Table 2.6. Mean (SD) baseline, follow-up (post-dosing) and relative change in $ppFEV_1$ grouped into baseline $ppFEV_1$ of low ($ppFEV_1$ 50-70%) or high ($ppFEV_1$ 70-90%) for both placebo and treatment groups (per protocol population) (†ANCOVA corrected values).

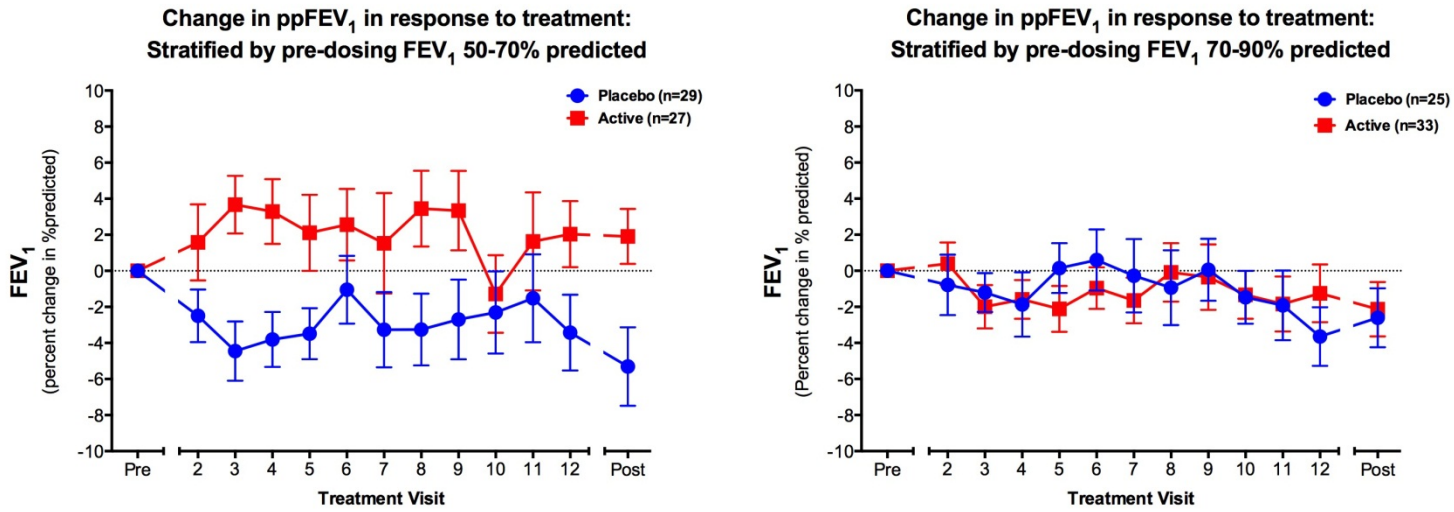


Figure 2.14. Change in mean FEV_1 in response to treatment with active gene therapy and placebo in per protocol population divided into baseline FEV_1 (i) LEFT - $ppFEV_1$ 50-70%; (ii) $ppFEV_1$ 70-90%. Error bars show standard error of the mean.

2.6.9.2 Subgroup analysis – change in LCI

A subgroup analysis of LCI based on the baseline severity of $ppFEV_1$ did not demonstrate any significant difference in treatment effect between either of the groups in both of the lung function ranges: (i) $ppFEV_1$ 50-70%: treatment effect = 0.05 (95% CI = -0.78, 0.69); (ii) $ppFEV_1$ 70-90%: treatment effect = -0.4 (95% CI = -0.88, 0.04) (Figure 2.15).

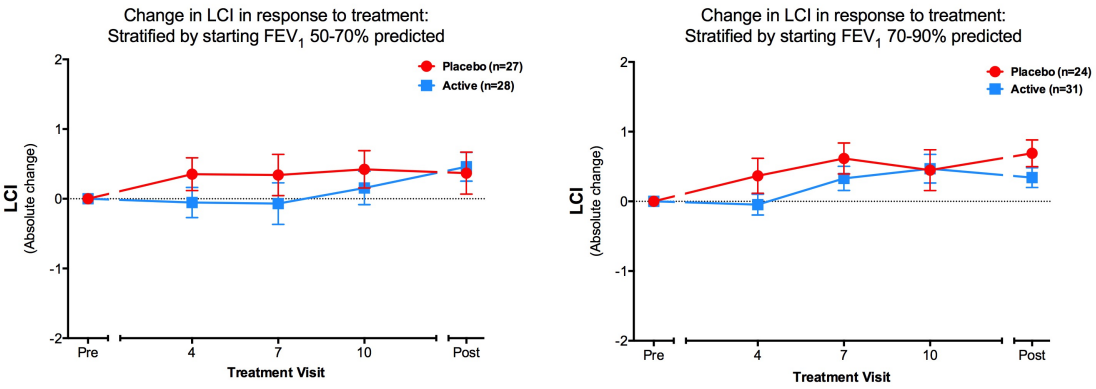


Figure 2.15. Change in mean absolute LCI in response to treatment with active gene therapy and placebo in per protocol population divided into baseline FEV_1 (i) LEFT - $ppFEV_1$ 50-70%; (ii) $ppFEV_1$ 70-90%. Error bars show standard error of the mean.

2.6.9.3 Subgroup analysis – CT scans for treatment efficacy

CT parameters in the lower FEV_1 subgroup demonstrated improvements in all CT indices following active treatment compared with the placebo group where parameters showed small deteriorations. All CT indices in the higher FEV_1 subgroup demonstrated no difference in treatment effect between placebo and active therapy. The results are summarised graphically in Figure 2.16.

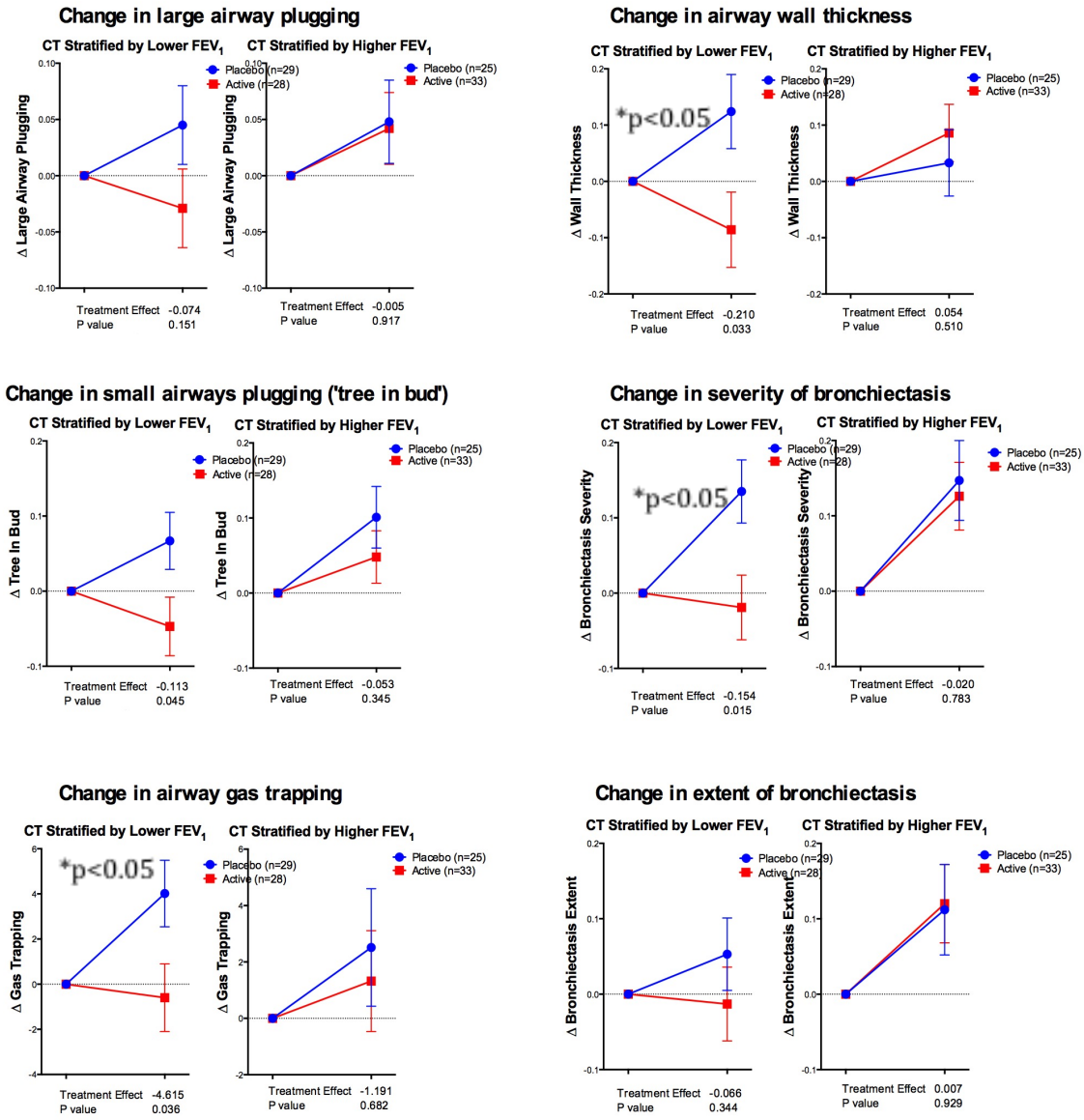


Figure 2.16. Graphs demonstrating changes in CT parameters (individually labelled) in lower and higher FEV_1 subgroups in response to placebo and active treatment; corresponding treatment effect and P value (ANCOVA corrected data) are shown. (*p<0.05)

2.6.9.4 Subgroup analysis – Quality of Life

QOL showed improvement in the active treatment compared with placebo groups in the more severe FEV_1 group in both QOL parameters (physical functioning and respiratory symptoms; p=0.48 and p=0.87 respectively); no treatment effect was seen in the less severe subgroup in the QOL physical functioning domain, yet an improvement was recorded in their respiratory symptoms (Figure 2.17).

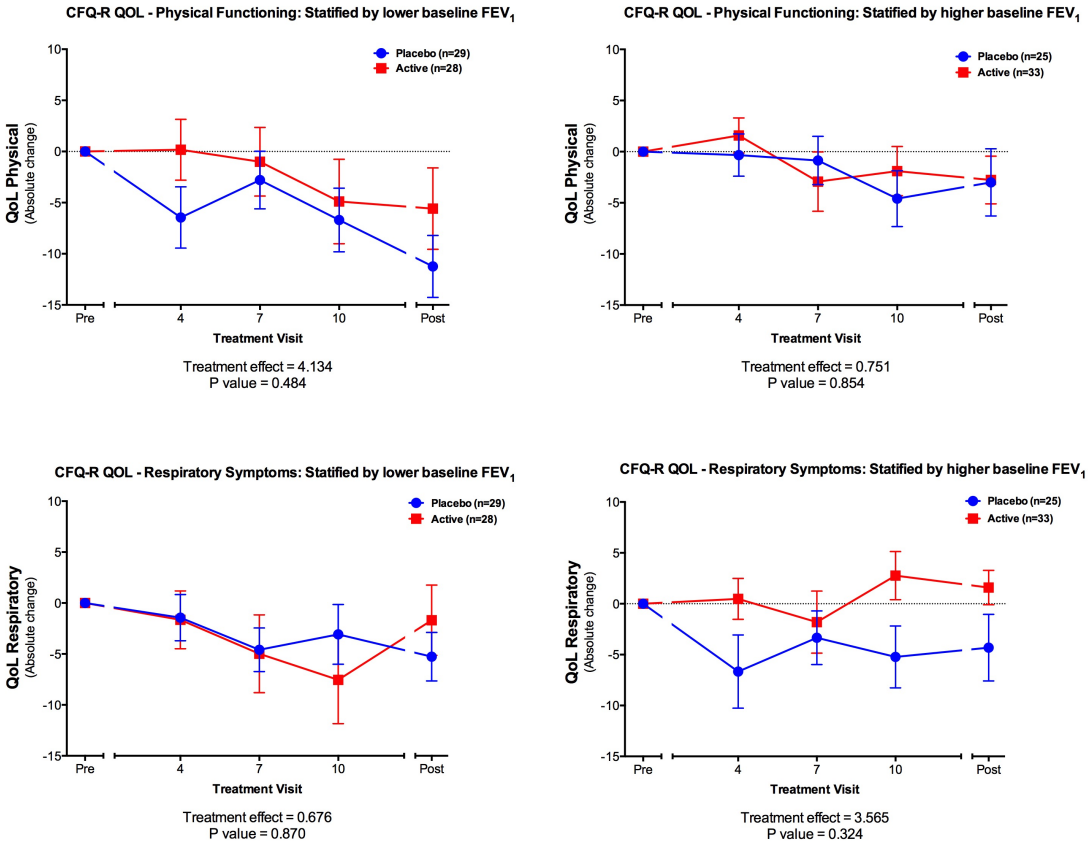


Figure 2.17. Graphs summarising the changes in CFQ-R QOL in the physical functioning and respiratory symptom domains, from both FEV_1 severity subgroups.

2.6.9.5 Summary of the FEV_1 severity subgroup outcome measures

To summarise and compare the results of all the main secondary outcome measures by FEV_1 severity subgroup, the data have been standardised and plotted in a Forest plot (Figure 2.18) which demonstrates a trend towards a treatment effect from gene therapy in all outcome measures in the more severe (lower FEV_1) subgroup, but no difference in treatment response in the less severe (higher FEV_1) subgroup. Whilst some of the responses are statistically significant, subgroup data were not statistically powered owing to the small number of subjects and little weight should be placed on the quoted p-values.

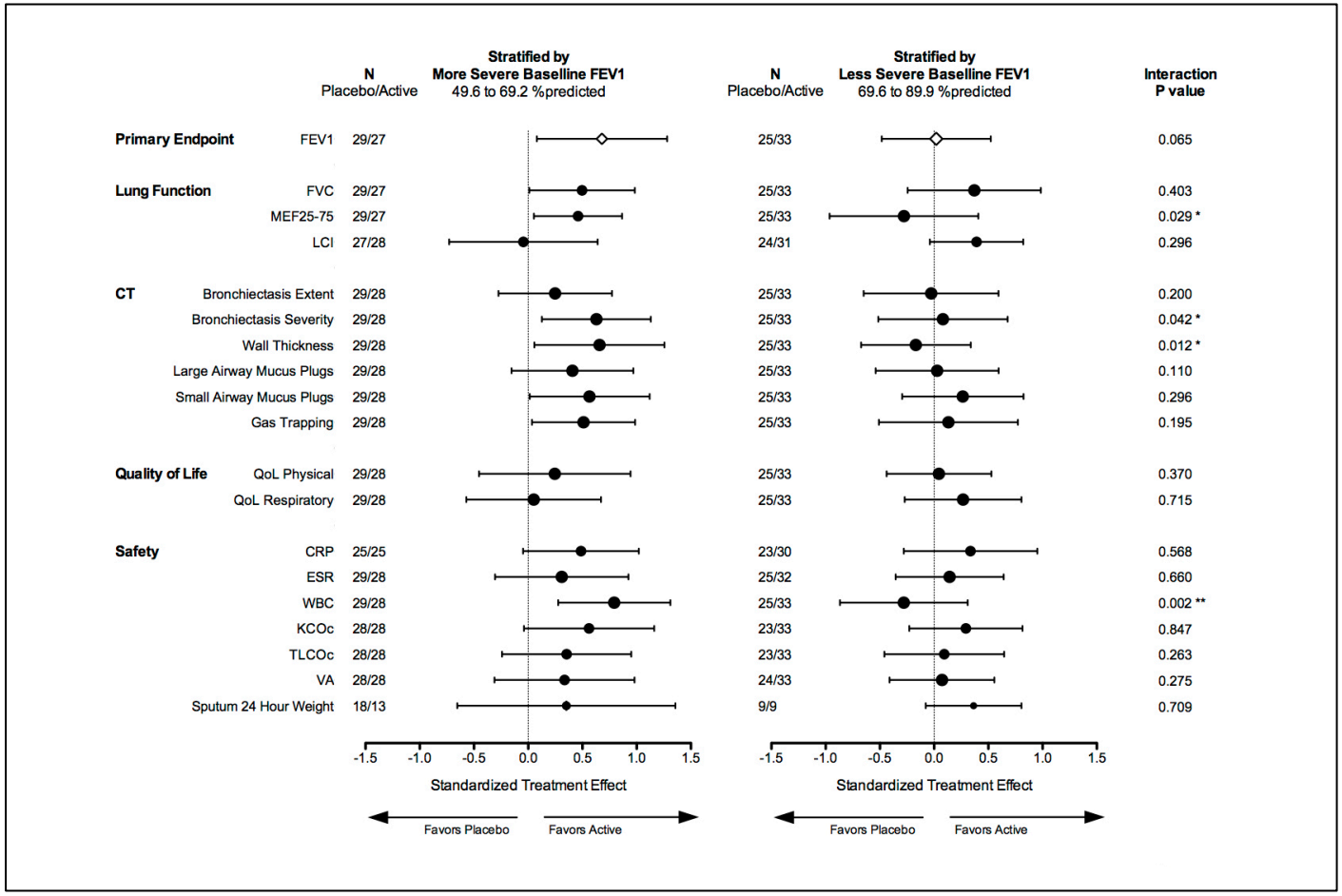


Figure 2.18. Forest plot of FEV_1 severity subgroup analysis, summarising the primary and secondary trial outcomes, including safety indices, in response to placebo and active treatment. Shown are the mean (95% CI) standardised treatment effects, with the size of each circle indicating the number of subjects represented.

2.6.10 Frequency and duration of pulmonary exacerbations

To assess whether gene therapy had a positive or detrimental effect on pulmonary exacerbations, data obtained from InForm and clinical research folders were analysed to identify (i) the total number and duration of antibiotic courses each group of patients received, and (ii) the time to first pulmonary exacerbation following dose 1.

2.6.10.1 Total number of antibiotic courses

There was no statistical difference between the total numbers of courses of oral or IV courses of antibiotics administered for pulmonary exacerbation between the two treatment groups (paired t test – oral: p=0.30; IV: p=0.33) (Table 2.7).

	Oral Antibiotics		IV Antibiotics	
	Placebo (n=54)	Active (n=62)	Placebo (n=54)	Active (n=62)
Number of antibiotic courses, n (%)				
0 courses	8 (14.8)	6 (9.7)	36 (66.7)	41 (66.1)
1 course	12 (22.2)	17 (27.4)	9 (16.7)	8 (12.9)
2 courses	10 (18.5)	11 (17.7)	5 (9.3)	10 (16.1)
>2 courses	24 (44.4)	28 (45.2)	4 (7.4)	3 (4.8)

Table 2.7. Number of patients taking courses of oral and intravenous antibiotics, by treatment group (per population cohort).

2.6.10.2 Total duration of antibiotics used

There was no statistical difference between the numbers of days oral or IV antibiotics were taken between the two treatment groups (oral: p=0.50; IV: p=0.97; Mann-Witney U) (Table 2.8 and Figure 2.19), suggesting that gene therapy neither reduced the frequency or duration of pulmonary exacerbations, nor increased it.

	Oral Antibiotics		IV Antibiotics	
	Placebo (n=54)	Active (n=62)	Placebo (n=54)	Active (n=62)
Total number of days of antibiotic taken (days)	1780	2190	539	665
Total number of days of antibiotic taken per patient (days)	33.0	35.3	10.0	10.7
Median (IQR) duration of antibiotic taken (days)	29 (14 – 45)	30 (15 – 57)	0 (0 – 15)	0 (0 – 22)

Table 2.8. Total number (median, IQR) of days of antibiotcs taken for oral and intravenous antibiotic courses, per treatment group (per population cohort). (Oral: p=0.50; IV: p=0.97; Mann-Witney U)

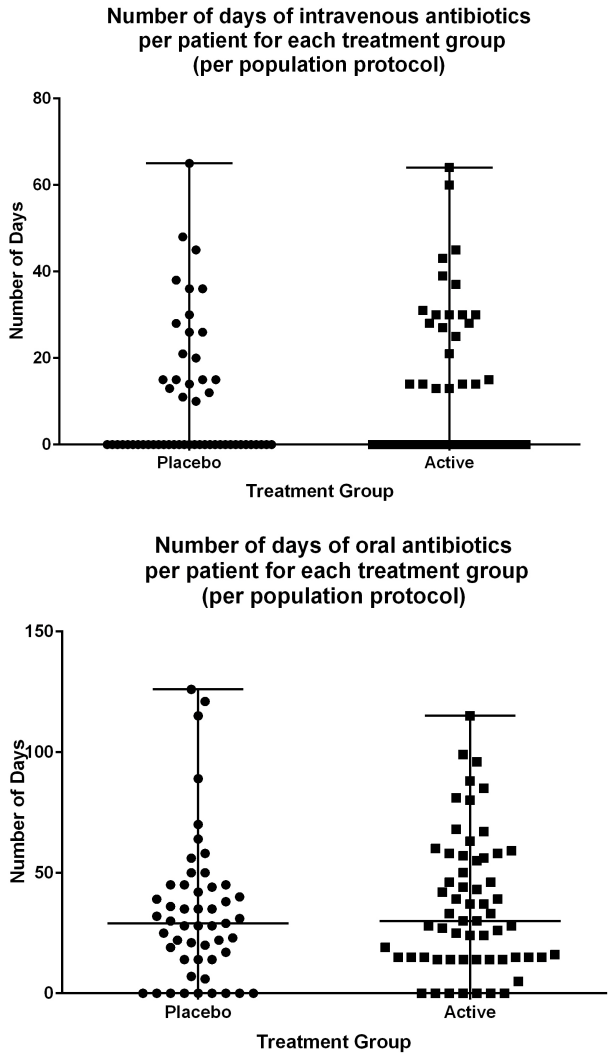


Figure 2.19. Plot of total (median/range) number of days of oral and intravenous antibiotic courses by individual patients number by each treatment group (per population cohort).

2.6.10.3 Number of days of antibiotics and the change in ppFEV$_1$

To investigate whether changes in lung function (ppFEV$_1$) were a result of multiple treatment courses with oral and IV antibiotics, rather than from gene therapy a correlation between each parameter was made. The results (Figure 2.20) demonstrate no correlation between antibiotic use by either route and change in lung function (oral antibiotics: placebo r=0.11, p=0.44; active r=-0.09, p=0.49. IV antibiotics: placebo r=0.05, p=0.74; active r=-0.03, p=0.80); the graphs visually demonstrate that there were no differences in the improvements or

deterioration in lung function, supporting that antibiotic use was not a confounder for overall treatment effect.

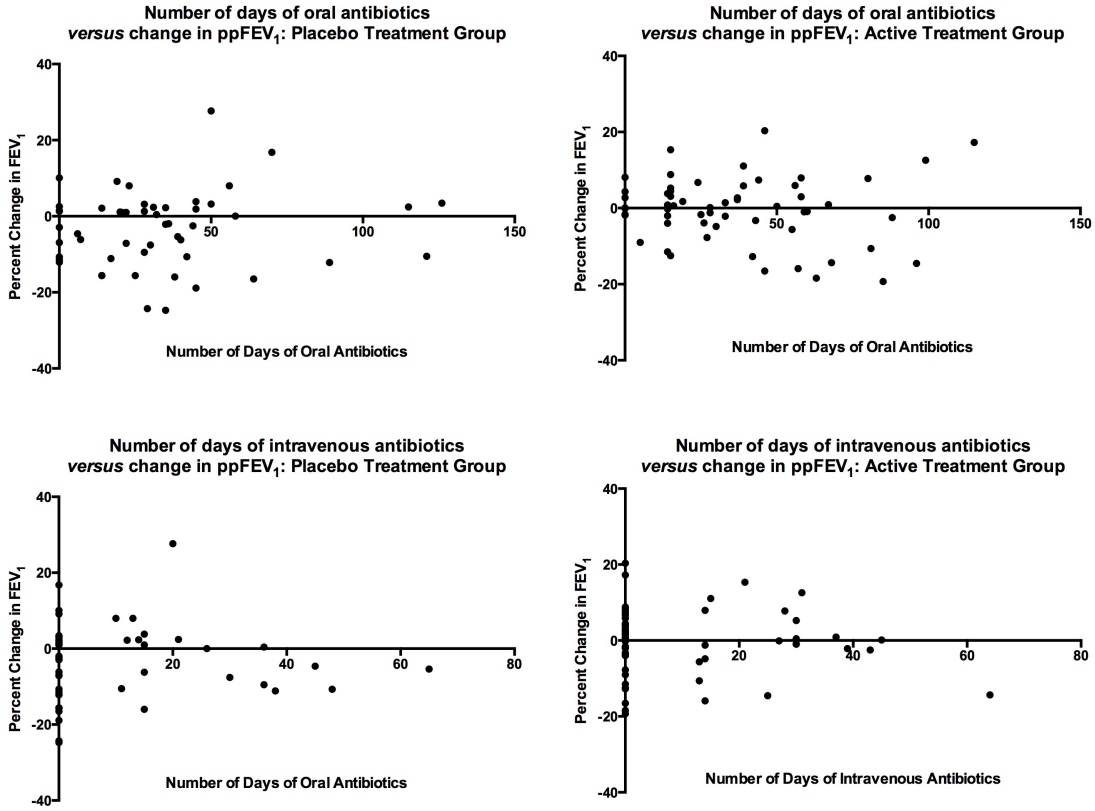


Figure 2.20. Total number of days of antibiotics against the change in $ppFEV_1$ in both treatment arms. TOP – oral antibiotics: (i) LEFT – placebo group (r=0.11, p=0.44), RIGHT – active group (r=-0.09, p=0.49); BOTTOM – IV antibiotics: (iii) placebo group (r=0.05, p=0.74), (iv) RIGHT – active group (r=-0.03, p=0.80).

2.6.10.4 Time to first pulmonary exacerbation

Time to first pulmonary exacerbation, as defined by the administration of a non-elective course of oral or IV antibiotics following dose 1, was not statistically different between treatment groups (p=0.38, Mantel-Cox), with a median survival 93 days (active group) and 114 days (placebo group). Thus treatment with gene therapy neither reduced the rate of exacerbation or the exacerbation free period. A Kaplan-Meier survival graph (Figure 2.21) shows the time to first

antibiotic use (pulmonary exacerbation) for both treatment arms, with no statistical difference between the two curves.

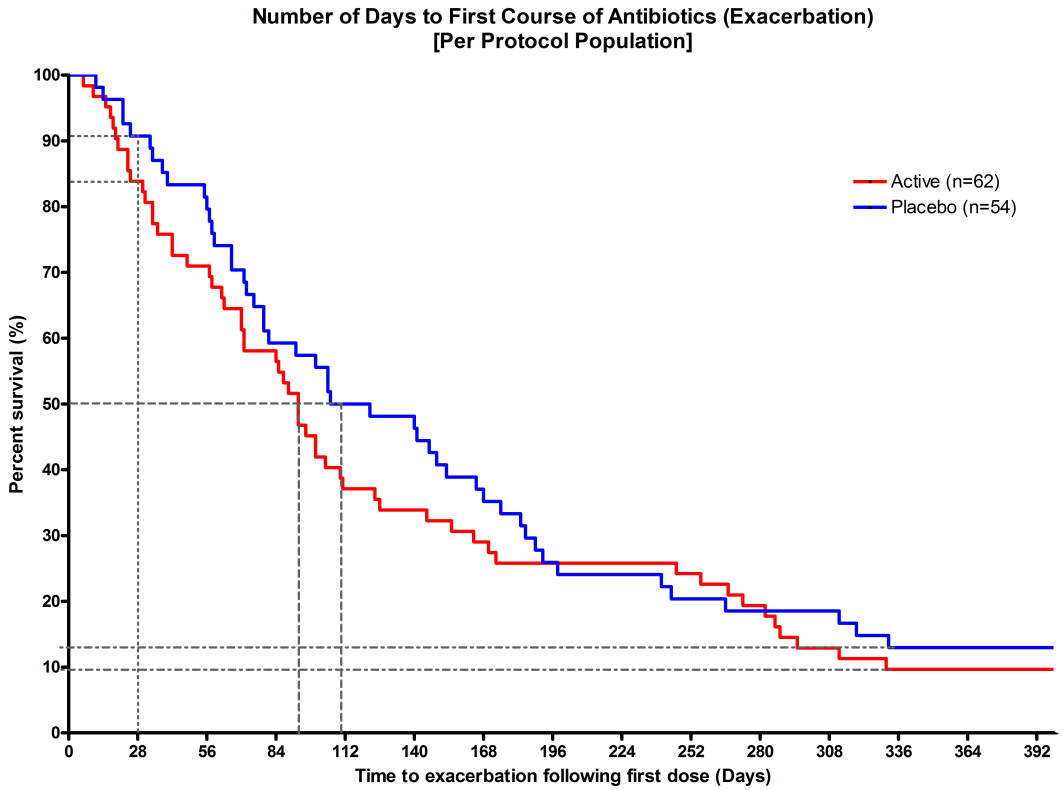


Figure 2.21. Kaplan-Meier graph of both treatment groups demonstrating the time to first pulmonary exacerbation (first antibiotic usage). Dashed lines are shown at 28-days post-dose 1, at 50% survival and at the end of the study.

2.6.11 Adverse events

The following table (Table 2.9) lists the 9 AE groups, and the (i) total number of adverse events, and (ii) total number of AEs per patient, per treatment group (per protocol population). No statistical difference was found between the total number of adverse events recorded between the treatment groups (p=0.68, Mann-Whitney U test) or total per patient (p=0.78, Mann-Whitney U test), supporting that treatment with gene therapy did not have a greater adverse profile over standard care and placebo.

	Placebo (n=54)		Active (n=62)	
	Total Number of AEs	Total AEs per patient	Total Number of AEs	Total AEs per patient
Lower Airway Respiratory Symptoms	429	7.9	556	9.0
Gastrointestinal Symptoms	112	2.1	112	1.8
Fever or 'flu-like Symptoms	60	1.1	84	1.4
Headache	63	1.2	69	1.1
Elevated Liver Function Tests	17	0.3	25	0.4
Upper Respiratory Tract Symptoms	123	2.3	210	3.4
Haematuria	9	0.2	14	0.2
Isolated Rise in Inflammatory Markers	44	0.8	42	0.7
Other	173	3.2	204	3.3
Total Adverse Events	**1030**	**19.1**	**1316**	**21.2**

Table 2.9. Table summarising the total number of adverse events and total AEs per patient, grouped into pre-defined AE categories, per treatment group.

2.6.11.1 Serious adverse events

6 SAEs were reported throughout the entire study, all of which were in the active treatment group. Each SAE was reported to the study sponsor; on the basis that all SAEs occurred in the active treatment group, the Trial Steering Committee were informed, who agreed that the events were all unrelated to the study medication. These SAEs are summarised in Table 2.10.

	Time from last dose	Subject (Age/Sex)	Description of SAE
1	4 weeks	47 Male	**Admitted to hospital with pancreatitis.** Pancreatic sufficiency. Previous episodes of non-alcoholic/ non-gallstone pancreatitis. Managed conservatively
2	5 weeks	28 Male	**Admitted with to hospital for within 24 hr of follow-up bronchoscopy** with severe headache, fever, pulmonary exacerbation and new isolate of *MRSA.* Treated with as an inpatient with IV antibiotics.
3	4 days	23 Female	**Iatrogenic pneumothorax** during the removal of an indwelling intravenous access device (Portacath®). Semi-elective admission for surgical removal and replacement of Portacath®. Small pneumothorax occurring during surgery – managed conservatively.
4	20 days	46 Female	**Post-surgical infection.** Elective Nissen's fundoplication for severe reflux. 10 days post-operatively admitted with fever and abdominal pain secondary to surgical site abcess.
5	11 days	25 Female	**Admitted to hospital with headache, vomiting and viral URTI symptoms.** In-patient observation overnight with conservative management.
6	16 days	14 Male	**Admission to hospital with mild vomiting.** Diabetes. Admitted for management of diabetic control during illness.

Table 2.10. Summary table of serious adverse events reported during the Multidose Trial.

2.7 Discussion

This chapter has summarised the culmination of over a decade's work from the UKCFGTC, and describes the first ever clinical trial of the repeated application of non-viral gene therapy (pGM169/GL67A) to patients with CF. The trial fulfiled its primary outcome, demonstrating a significant treatment effect on relative change in $ppFEV_1$, albeit as a result of stalilisation in lung function with active treatment combined with a decline in lung function in the placebo group. The results presented in this chapter have focused on patient recruitment and

retention, safety, and on treatment efficacy. This chapter will discuss each of these and the conclusions that can be made from this study.

2.7.1 Patient recruitment and retention

The study was powered to complete with 120 patients, and to allow a margin for the withdrawal of patients, a target of at least 130 subjects was set for recruitment. In itself, for a two-site, non-commercial study, this number of subjects is sizeable; owing to the discrepancy in facilities available and subgroup involvement between the London and Edinburgh sites, it had been agreed that London would recruit a larger proportion of patients. This study illustrates the difficulties in recruiting and retaining large numbers of patients with a rare disease, and maintaining their 'enthusiasm' for visits over a prolonged study period. At the same time, it illustrates that a dedicated and motivated research team that was organised and engaged well with patients could successfully recruit and retain patients from the RBH and from around the U.K.

It had initially been planned that recruitment at the London site be entirely from RBH, but this yielded a significant shortfall in patient numbers. This was largely due to patients failing to meet the inclusion criteria, being deemed clinically unstable by the CF clinical team, being enrolled on other interventional studies, and by the time commitments required for the study being unfavourable to potential subjects. Furthermore, owing to a slower rate of recruitment in Scotland, London agreed to recruit extra patients to address the shortfall.

Because recruitment was slower than anticipated, the initial date to close recruitment was extended following approval of the funder, and a lengthy process to activate and recruit from CF centres throughout the UK was initiated to ensure that the study was not underpowered. With this effort, 136 patients were randomised to treatment. It was a great achievement that patients geographically scattered throughout the UK would make monthly journeys to London, with some patients travelling journeys of over 200 miles (Leeds) and over 4 hours in each direction.

A total of 14.7% of patients withdrew from the study (having received at least one dose of gene therapy or placebo). Whilst more than twice the number of patients withdrew from the gene therapy group compared with the placebo group (18.4% *v* 10.0%), this finding was not statistically significant and should be taken in context that more active patients were enrolled into the study. On further analysis of reasons for withdrawal, neither the author, the Consortium nor the Trial Steering Committee identified any concerns that could be treatment-related. It is therefore felt adequate to explain this by random chance and the increased number of patients in the active group.

Other than patients unavoidably acquiring a trial exclusion criterion (e.g. *M. abcessus* or *MRSA* infection), patients requested to withdraw from the study in very few cases (n=7) owing to time and travel commitments involved in the study. During the course of the study, ivacaftor was licensed for the *G551D* mutation with 7 subjects being eligible for its use and, whilst all subjects were counselled, only 3 elected to withdraw from the MDT; the other 4 completed the trial. This exemplifies the commitment of some CF patients and their motivation to forwarding the science and treatment of the disease.

The study was completed with 116 patients receiving 9 or more doses (with 114 patients having paired measurements for FEV_1); the study was therefore short by 4 subjects to reach the statistical power required to detect changes in FEV_1, however this power calculation was conservative as covariant adjustment of data would be expected to increase statistical power.

2.7.2 Improvements in ppFEV$_1$ in response to gene therapy

The Multidose Trial is the first to clinically demonstrate a significant treatment effect, in response to non-viral gene therapy (pGM169/GL67A), recording a relative improvement in $ppFEV_1$ of 3.7% following 12 months of treatment. In the active treated population as a whole, stability in lung function was

predominantly observed with the major treatment effect resulting from a fall of 4.9% in the placebo arm. It is of relevance to highlight the difference observed in the decline in lung function between the higher and lower baseline FEV_1 placebo groups, 2.6% and 5.3% respectively. A reduction in FEV_1 in a placebo group has been recognised in other prospective clinical trials in CF [111, 115, 138]. There is an inherent, background natural decline in lung function which is recorded in registry data from the CF population: approximately 1-2 % decline per year [2, 192], and therefore the values reported in the overall MDT population and those with more severe lung disease demonstrate a greater than expected decline. One explanation is that CF registry data are collected during periods of clinical stability and it is often the case that more severely affected patients fail to provide data to these calculations as they remain clinically unstable, leading to an underestimate of the true value in a heterogeneous CF population. As the decline in lung function seen in the MDT has been recorded during 'mixed' levels of health and stability throughout the trial, this may inaccurately correlate with the registry data.

A further possible reason for this fall in lung function is that patients at entry into a clinical trial may have optimised themselves by strict treatment adherence to ensure they pass screening. It is likely that during the course of a trial, the patient cohort relapses to normal behaviour with a reduction in treatment compliance and a concomitant fall in lung function. Possible explanations for a greater decline in lung function seen in patients with a lower FEV_1 include (i) patients with more severe lung disease may naturally decline at an accelerated rate compared with patients with milder lung disease owing to increased airways inflammation and rate of pulmonary exacerbations; (ii) patients may be less compliant with medication than patients who have preserved their lung function [193].

Despite the entire treatment cohort failing to show any improvement from baseline, the maintenance of stable lung health in CF is in itself invaluable long-term.

To put in context the treatment effect observed with gene therapy in relation to licensed CF medications used in daily practice, this result is recognised as modest. Mucolytics and oral (azithromycin) and nebulised antibiotics each demonstrate approximately a 7% improvement in ppFEV$_1$. This treatment effect is significantly lower that that seen with the CFTR potentiator ivacaftor in patients with the *G551D* mutation (relative treatment effect = 16.8%) [123], and lower than that seen with the CFTR corrector lumacaftor 400 mg (in combination with ivacaftor) in *Phe508del* homozygous patients, who demonstrated a relative change in FEV$_1$ of 4.8%; this trial also demonstrated a reduction in pulmonary exacerbations [132].

2.7.3 Improvements in other measures of airway physiology

Small improvements, corresponding to those seen in FEV$_1$, were reported in other measures of large and small airway function, such as LCI and those not discussed within this thesis (e.g. FVC and alveolar gas transfer; Figure 2.13), following 12-months of nebulised gene therapy. These are summarised and presented as standardised treatment effects in a forest plot (Figure 2.13), which shows a trend in favour of treatment with gene therapy throughout all airway parameters, when compared with placebo. None of these outcomes measures was statistically powered to detect changes, and therefore whilst these changes were small and not significant they support a trend towards improvement in the CF airways with gene therapy.

Small differences between the active and placebo groups were also noted in the CT images of the lung, with a trend towards improvements in the airway parameters in the treated population, principally within the smaller airway component (gas trapping and small airway mucus plugging). Relatively fixed changes to airway pathology such as bronchiectasis, which by definition is irreversible and would be unexpected to improve, suggested slowing of bronchial wall damage in response to treatment, however it is possible that a 12

month period is too short to appreciate any improvement.

It is suggested that the small degree of airway change detected, was insufficient for patients to notice a clinical difference, this being reflected in the minimal changes observed in patient-reported quality of life measures, with any changes being non-significant and failing to reach the MICD.

The study was not powered to detect differences in other outcome measures, though FVC and gas trapping achieved statistical significance. However, results suggest a trend that nebulised non-viral gene therapy administered monthly can improve airways disease.

2.7.4 Improvements in airway physiology in cohort subgroups

A striking difference in treatment effect was seen when patients were separated into subgroups based on their baseline ppFEV$_1$ (higher (70-90%) and lower (50-70%)), with the severe cohort demonstrating a statistically significant treatment effect of 6.4% ppFEV$_1$ with gene therapy, whilst that of the milder cohort established no difference between active and placebo treatment. This trend was seen throughout all parameters of airway physiology discussed within this thesis.

Data are presented comparing treatment effect with antibiotic usage, based on the fact that patients with lower lung function are more likely to exacerbate and require antibiotics [194], which might have improved lung function. These data failed to identify a relationship between antibiotic use and treatment effect, and making antibiotic use less likely to have caused the treatment effect observed.

It is suggested that a higher proportion of active product is retained within the larger airways in the more severe subgroup, leading to increased gene transfer and thus expression. As FEV$_1$ is largely a measure of the proximal airways, this is possibly reflected in the changes seen within this cohort. The retention of gene

therapy is based on airway deposition scans performed during the Run-In Study (Chapter 1, section 1.13.2), where the fractional deposition of Technetium-99m labelled human serum albumin following nebulisation into the CF airway was twice as great in patients with an $ppFEV_1$ 50-70% compared to 70-90% (6.0% compared to 2.9% fractional deposition to 2^{nd}-8^{th} generation bronchial airways respectively).

It is further hypothesised that increased airway inflammation in the more severe CF cohort is likely be associated with an increased rate of cell proliferation, and in turn a less intact nuclear membrane, thus making the nucleus more accessible to gene transfer [195].

2.7.5 Patients missing treatment doses

A significant difference is seen (section 2.6.3) between the numbers of doses of treatment (both gene therapy and placebo) missed in the first half (doses 1 – 6) of the study compared with the second half (doses 7 – 12), with significantly more doses being missed in the latter period. As there was no difference between the doses missed between the treatment groups, nor a difference in total adverse events between the groups, the author would conclude that this effect is not related to adverse effects from gene therapy.

The Consortium confirmed that missed doses did not occur seasonally, i.e. in winter months compared with summer (data not presented), suggesting that this effect did not relate to vulnerable periods when patients were more likely to experience a pulmonary exacerbations.

As patients were well and clinically stable at baseline, it is possible that over the 12 month period the level of natural stability has declined, resulting in patients being clinically too unwell to dose; it is also possible that, owing to the length of the study, less engagement by the trial subjects reduced attendance for dosing as

it is not possible from the trial source data to tease out specific reasons for omission of dosing.

The protocol allowed patients to miss up to and including 3 doses of gene therapy. It could be hypothesised that regular monthly dosing with gene therapy could have an additive effect on the airway epithelium, with benefits from the previous dose remaining present when the next dose is received.

It is possible that patients missing doses could have lost any additive effect from the previous month's treatment and regressed towards their baseline; this is more likely to be clinically significant when patients missed consecutive doses (2 or 3) and potentially underestimate a '12-dose' response, in effect diluting the any overall treatment effect in the active group.

2.7.6 Choice of dose and dosing interval

Whilst it is probable that, for example in patients missing doses, extending a dosing interval from 28 days to 56 or longer is likely to reduce efficacy, it is possible that the chosen dosing interval of 28 days may also be too long. It may be that shortening the dosing interval might provide longer gene expression within the airways and increase treatment efficacy.

Furthermore it may be possible that the dose of gene therapy used (5 ml) is less efficacious than a higher dose. This dose was chosen from the Pilot Study, when it was concluded that it was safe, provided efficacy and was associated with the least systemic side effects that would risk unblinding in the Multidose study. It is arguable whether 'one dose fits all', referring to nebulising 5 ml of gene therapy to patients of all sizes, for example a child of 12 yr with a small lung surface area, compared to that of a tall adult male. Within the small numbers of the study, in particular children, this analysis was not undertaken, but it is for consideration that dose adjustments could perhaps be made based on predicted airway surface areas. It is therefore suggested that increasing the dose of gene therapy could

provide further gene transfer to the airways and additional clinical benefit. Further studies using the current pGM169/GL67A complex should attempt to optimise dosing efficacy, by increasing dose, adjusting the dosing frequency, or with the addition of a CFTR-poteniator to augment *de novo* CFTR function.

2.7.7 Pulmonary exacerbations

Studies of conventional CF medications have recorded reductions in pulmonary exacerbations in parallel to their benefits on lung function. It could be hypothesised that in the MDT, restoring CFTR function would ameliorate the airway function by improving hydration and correcting the pH of the ASL, facilitating MCC and restoring innate immune responses.

The MDT did not formally capture pulmonary exacerbations as an outcome measure but instead recorded the number of courses of antibiotics a surrogate marker. The MDT failed to demonstrate a reduction in the frequency of pulmonary exacerbations in response to gene therapy, with no differences between the numbers of courses of antibiotics taken between the two treatment groups; at the same time, active treatment did not increase the exacerbation frequency that could have indicated adverse effects of treatment on the airway. Furthermore, there was no significant difference between the time to first exacerbation between the two treatment groups (Figure 2.21). In future clinical studies, it would be advantageous to use pre-defined criteria to define pulmonary exacerbations as has been the case in previous interventional CF studies [182], in attempt to reduce any under- or over- reporting of pulmonary exacerbations.

2.7.8 Safety profile of gene therapy

The trial demonstrated a good safety profile and was reviewed at intervals by the DSMB. The overall number of adverse events was similar and not statistically different between gene therapy and placebo treatment, although slightly more

respiratory adverse events were reported in the active treatment group. Two patients exited the study due to adverse events directly cited as treatment reactions – one subject in the placebo group, and the other in the active group, who described 'flu-like symptoms after their first dose of gene therapy. However, it was likely this was significantly exacerbated by a 400 mile round trip and an unpleasant train journey. 6 SAEs were reported, all in patients in the gene therapy arm – these were all reviewed by the Trial Steering Committee and agreed they none was related to gene therapy.

2.8 Conclusions

This chapter describes the background, the protocol and results of a pivotal trial in CF. In contrary to the hypothesis, improvements in lung function and a reduction in the frequency of pulmonary exacerbations following non-viral *CFTR* gene therapy (pGM169/GL67A) were not seen, and the chapter hypothesis should be rejected, however stability in lung function with active treatment was demonstrated. It is the opinion of the author that the aims of the study were sufficiently robust, and the number of trial participants adequate, to satisfactorily investigate this novel treatment in a phase II study. The trial however establishes the proof-of-concept that repeated application of non-viral *CFTR* gene therapy can safely lead to clinical benefit in terms of airway function. It was an unexpected finding that patients with more severe lung disease recorded a greater benefit from treatment and mechanisms for this have been proposed.

Gene therapy has the potential advantage over currently available CF medication that it is not mutation-specific and therefore limited to defined populations based on *CFTR* genotype, and could be administered on a less frequent basis than current daily treatment regimes thus reducing treatment times and improving quality of life.

The GTC has concluded, based on these data, that 5 ml of pGM169/GL67A administered monthly is not sufficient to produce the clinical improvements seen in other interventional studies to a level suitable for clinical use. It is likely, however, that this product and its delivery could be modified, with the possible addition of a CFTR potentiator, to optimise its function, in future clinical trials.

Chapter 3: Assessing Airway Epithelial *CFTR* Expression

This chapter will focus on the methods used within the MDT and PhD for making electrophysiological measurements of the upper (nasal) and lower airway epithelium, and for obtaining samples of airway epithelium to quantify transgene *CFTR*.

3.1 Measurement of Airway Epithelial Potential Difference

The first 1-2 cm of nasal mucosa is lined with non-keratinised stratified squamous epithelial cells, which then transitions into ciliated pseudostratified columnar epithelium and continues through to the lower airway [196].
Given the similarities between the ciliated epithelium of the upper and lower airway, assumptions may be reasonable, but there are sparse data comparing PD at the two sites directly. Data comparing upper against lower airway PD (LAPD) in 7 patients with chronic obstructive pulmonary disease failed to identify a correlation between the two organs, reporting that 3 (from 7) patients (ex-smokers) had lower chloride secretion from the lower airway than in the nose [197].

3.1.1 Measurement of transepithelial PD

Airway PD, measured at either site, can be considered as two systems: (1) a recording system, and (2) a perfusing system. Both involve placing a flexible catheter, attached to a recording electrode, against the airway mucosa and perfusing physiological solutions to block or stimulate ion transport; when compared with a submucosal reference electrode, a real-time measurement of transepithelial PD is achieved.

There are multiple advantages of taking measurement from the nose: it is easily accessible, is less invasive, carries fewer complications and allows for repeatable measurements to be taken; in direct contrast, those from the lower airway rely on the ability to directly access the bronchial epithelium.

3.1.2 Physiological (perfusing) solutions measuring airway PD

Physiological ('perfusing') solutions are formulated specifically either to block Na^+ absorption (via ENaC) or stimulate Cl^- secretion, predominately via CFTR although non-CFTR channels (e.g. calcium-activated chloride channels (CaCC)) can produce a transient ion efflux.

The following solutions are used in nasal and/or lower airway PD measurement, and are perfused sequentially in the following order. The compositions of all PD solutions are detailed in *Appendix B*.

3.1.2.1 Ringer's solution

Ringer's solution is a physiological saline solution and measures a non-stimulated basal PD that is thought predominantly to reflect Na^+ transport. Under basal conditions, the airway epithelium has a predominant absorption of Na^+ and thus produces a negative PD (when measured from the mucosal surface); owing to hyperabsorption of Na^+, people with CF have a more negative baseline than the non-CF population [198] (Figure 3.1). Ringer's solution is used in both NPD and LAPD, with responses to Ringer's solution being referred to as 'basal' PD within this thesis.

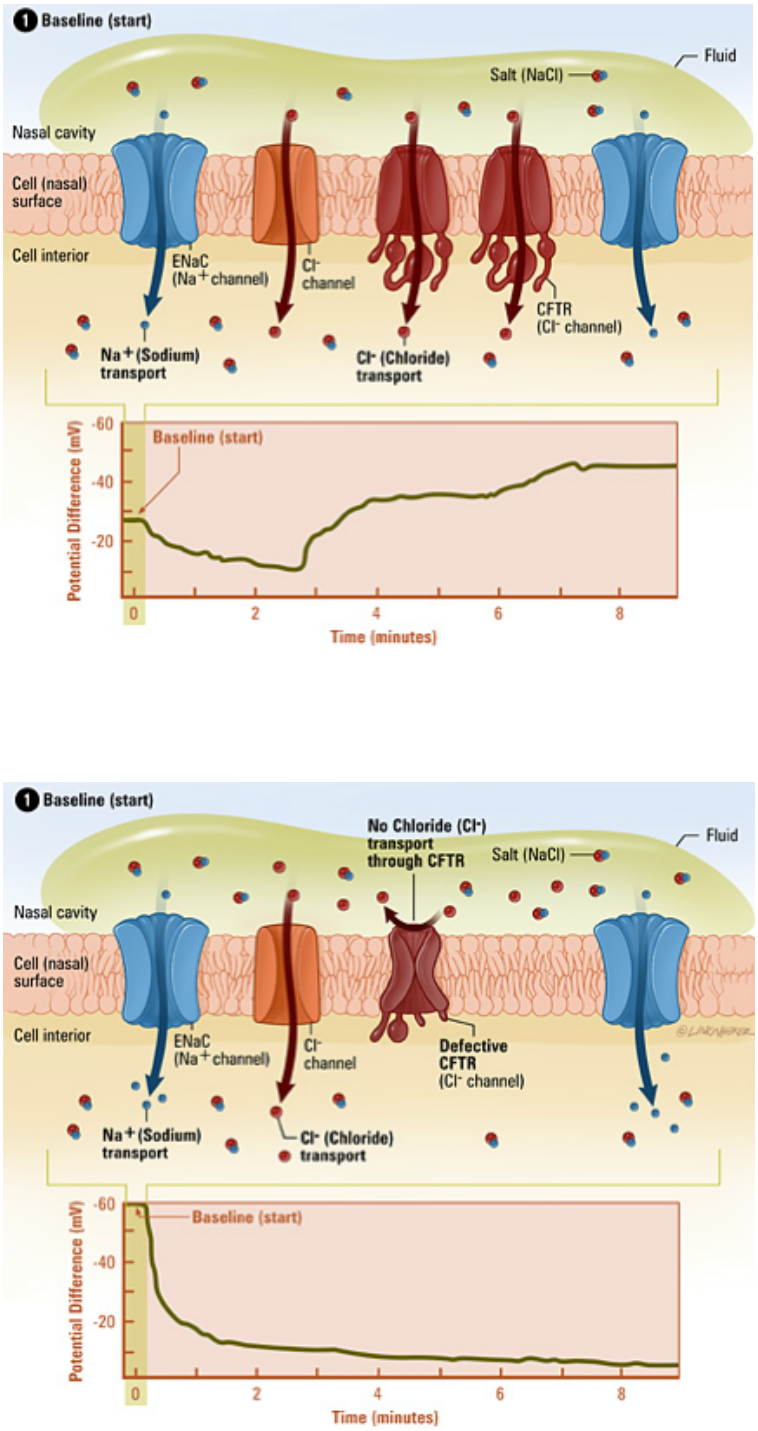


Figure 3.1. Diagram representing epithelial ion transport during perfusion with Ringer's solution, with the corresponding measured NPD (perfusion duration highlighted in yellow) in (i) TOP: healthy nasal epithelium; (ii) BOTTOM: CF nasal epithelium. (Reproduced from Johns Hopkins Online [199])

3.1.2.2 Ringer's solution with amiloride (0.1 mM) (RA)

Amiloride is a potassium-sparing diuretic, which acts directly to block ENaC. When taken systemically, it acts as a diuretic by preventing Na^+, and hence water, reabsorption in the renal tubules. When applied topically onto the nasal mucosa, amiloride similarly blocks ENaC and thus is used in NPD to characterise Na+ transport. Owing to the innate lack in ENaC regulation, an enhanced response to amiloride is seen in the CF population. These are demonstrated in Figure 3.2.

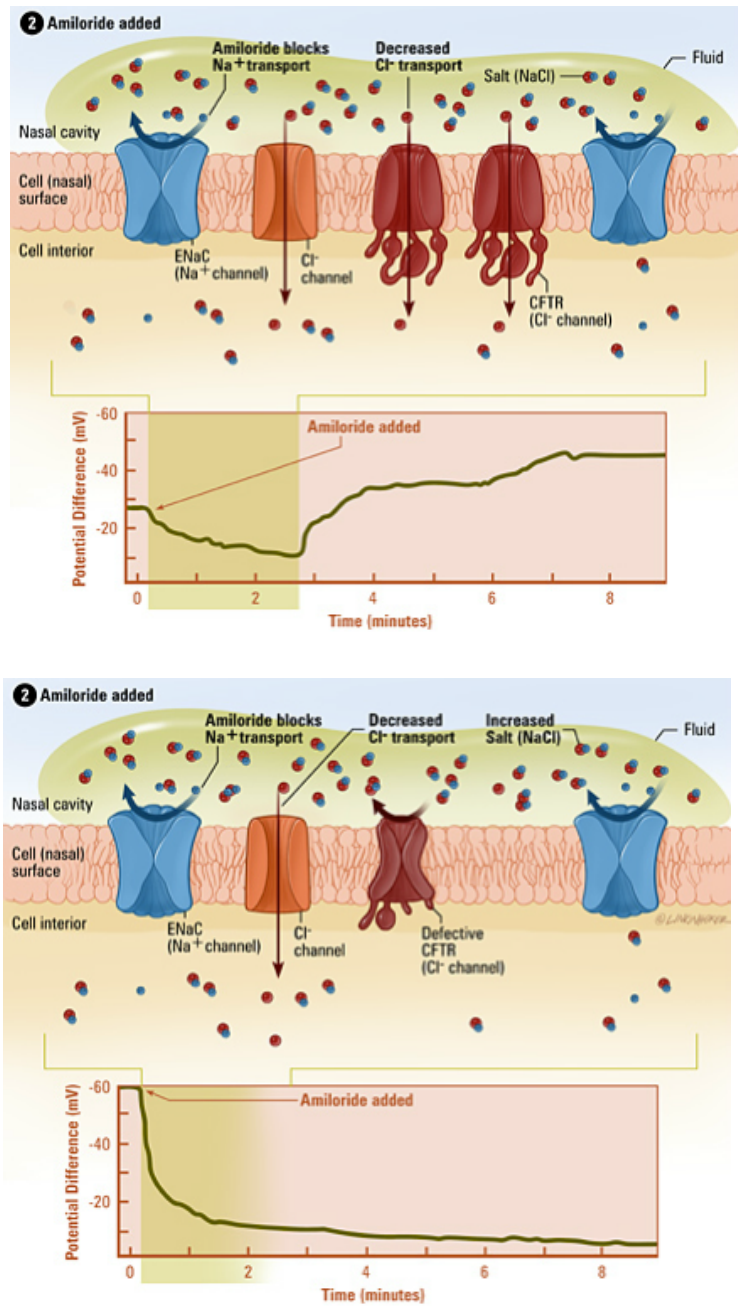


Figure 3.2. Diagram representing epithelial ion transport during perfusion with Ringer's + amiloride (RA) solution, with the corresponding measured NPD (perfusion duration highlighted in yellow) in (i) TOP: healthy nasal epithelium; (ii) BOTTOM: CF nasal epithelium. (Reproduced from Johns Hopkins Online [199])

3.1.2.3 Zero chloride solution with amiloride (0.1 mM) (ZCA)

A chloride-free solution induces passive (non-stimulated) Cl^- transport down a concentration gradient. In the healthy population, Cl^- moves out of cells through the chloride channels, including CFTR, making the mucosal cell surface more negative; this response is absent or blunted in CF, with an isoelectric (flat) or depolarisation (more positive) being observed. (Figure 3.3)

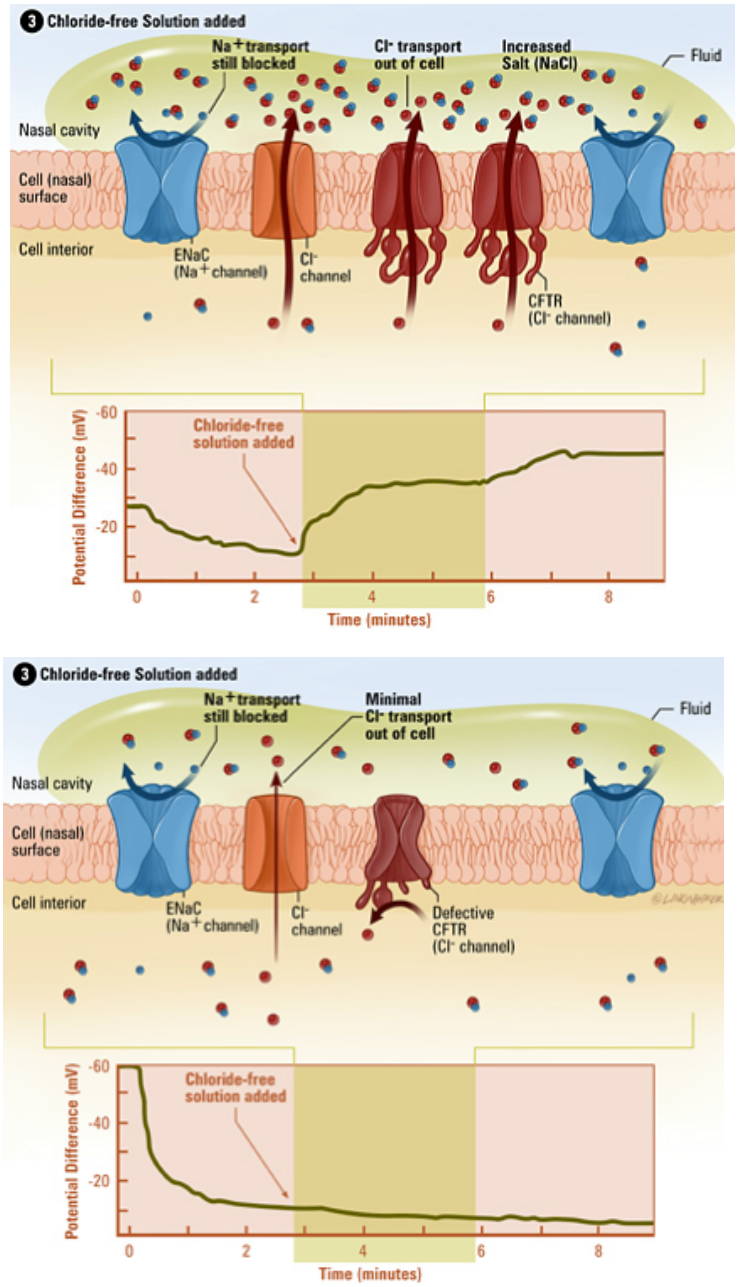


Figure 3.3. Diagram representing epithelial ion transport during perfusion with zero chloride + amiloride (ZCA) solution, with the corresponding measured NPD (perfusion duration highlighted in yellow) in (i) TOP: healthy nasal epithelium; (ii) BOTTOM: CF nasal epithelium. (Reproduced from Johns Hopkins Online [199])

3.1.2.4 Zero chloride solution with amiloride (0.1 mM) and isoprenaline (10 µM) (ZCAI)

Isoprenaline is a β1 and β 2-adrenergic receptor agonist that exerts its action by stimulating intracellular cyclic adenosine 3',5'-monophosphate (cAMP) production. As CFTR function is stimulated by cAMP [9], isoprenaline applied topically to the airway epithelium leads to active secretion of Cl^- , specifically through CFTR (active or stimulated Cl^- transport), which further depolarises the mucosal surface in the healthy population and makes the PD measurement more negative; again, no response is seen in CF (Figure 3.4).

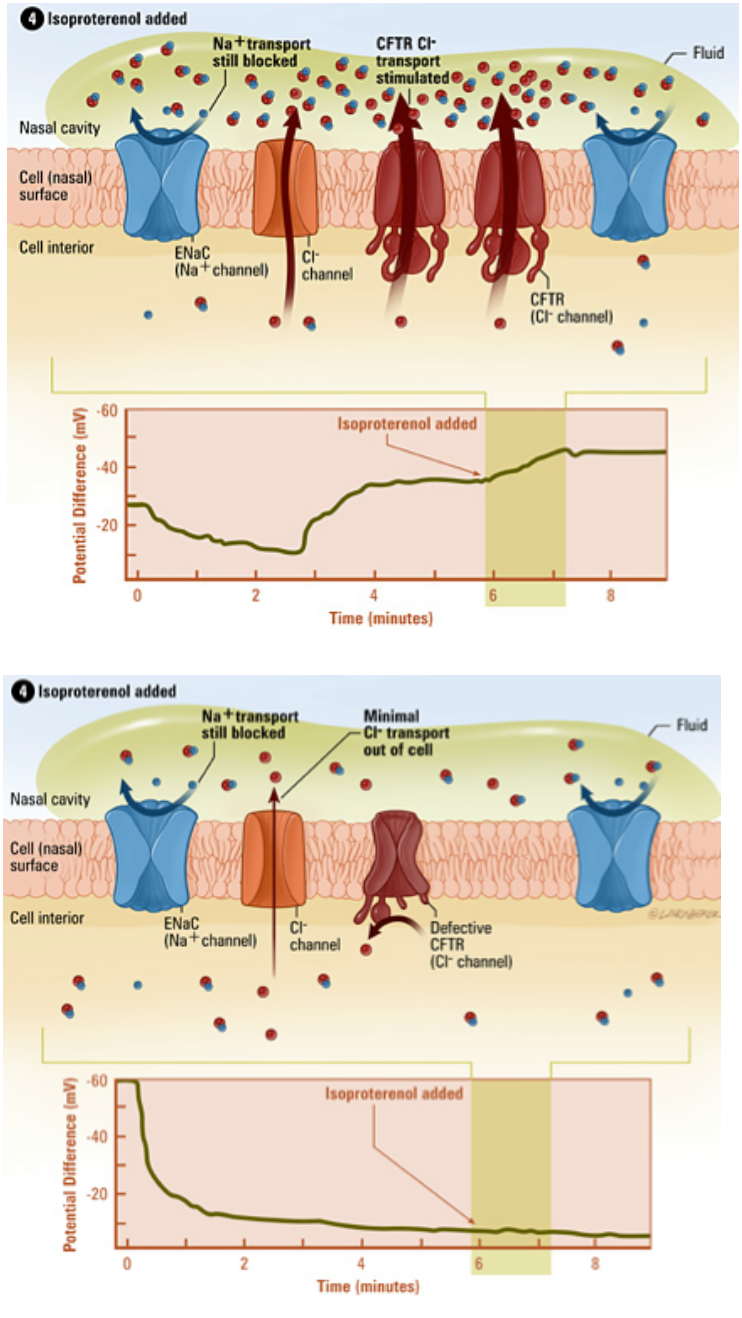


Figure 3.4. Diagram representing epithelial ion transport during perfusion with zero chloide + amiloride _ isoprenaline (isoprotolol) (ZCAI) solution ,with the corresponding measured NPD (perfusion duration highlighted in yellow) in (i) TOP: healthy nasal epithelium; (ii) BOTTOM: CF nasal epithelium. (Reproduced from Johns Hopkins Online [199])

Owing to the relatively short half-life of topical amiloride on the mucosa [200], it is essential that amiloride remain in both zero chloride solutions used in NPD to ensure that ENaC remains blocked, thus preventing hyperpolarisation of the PD from returning Na^+ transport.

3.1.2.5 Zero chloride + isoprenaline (10 μM) solution (ZCI)

As no gene therapy trial has ever demonstrated correction of sodium transport, and in order to minimise the volume of solution within the airway and to prevent pooling of endobronchial solutions that would alter PD measurements, it had been previously adopted in the LAPD SOP to: (1) omit the Ringers/amiloride step from the LAPD protocol; (2) combine the zero chloride and zero chloride + isoprenaline phase. For this reason, a zero chloride and isoprenaline solution with no additional amiloride has been formulated.

This chapter will now describe both techniques and their limitations.

3.2 Nasal Potential Difference

Nasal PD (NPD) is used to measure CFTR function in the nasal mucosa, clinically in cases of diagnostic uncertainty where CFTR dysfunction is suspected, and as a research tool [83] in clinical trials of new agents to restore CFTR function.

The original measurement and validation of airway PD was developed by Knowles *et al* [196] and Alton *et al* [72], and other than subtle refinements to the equipment, substrates and techniques used, the method in essence remains the same. Differences in techniques have been described, with regards to placement of catheter within the nasal cavity [201] and temperature of perfusing solutions [202]. NPD has been standardised within the Cystic Fibrosis Foundation Therapeutics Development Network (CFFTDN) [203] and European Cystic Fibrosis Society (ECFS) Diagnostic Network Working Group [204]. The technique

used in the the Department of Gene Therapy (Imperial College London) and RBH closely reflects that of the ECSF SOP, and was used within the Multidose Trial, and adapted as necessary for additional research projects within this thesis.

3.2.1 Performing nasal potential difference measurement

The following method explains how to set up an NPD perfusing and recording circuit, and how to measure the NPD. For clarity, the NPD set-up is shown in a photograph (Figure 3.5) and annotated diagram (Figure 3.6), but is explained in detail in the following section.



Figure 3.5. Photograph of a healthy volunteer having nasal potential difference measured. The photograph demonstrates a (left to right) laptop to record the PD, a voltmeter, 2 electrodes (reference and recording), nasal catheter (in the nasal cavity), a skin abrader, 4 syringe infusion pumps containing NPD solutions connected by 3-way taps and extension tubing.

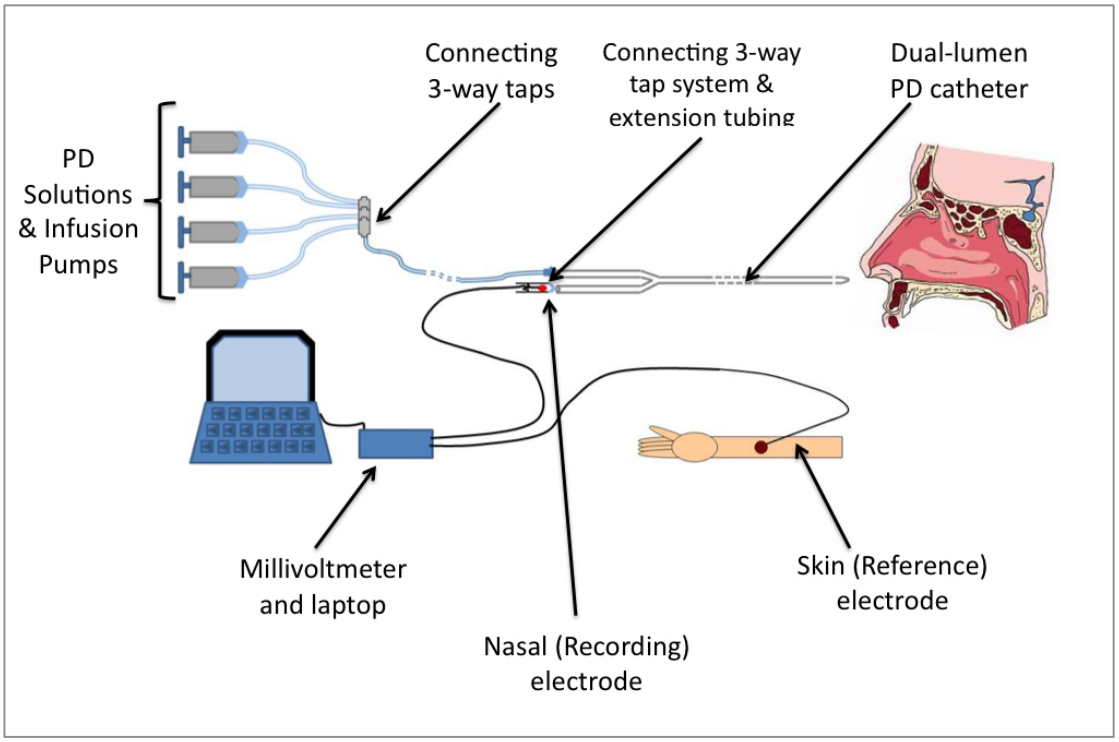


Figure 3.6. Schematic diagram showing the set-up of nasal PD equipment, as demonstrated in the corresponding photograph (above).

3.2.2.1 Double lumen catheter

The procedure involves the insertion of a double-lumen catheter into the nasal cavity. The catheter has two channels: a recording channel to measure mucosal PD and a perfusing channel to instil ionic solutions onto the mucosa. Whilst previously studies have used a modified 12F Foley urinary catheter, in keeping with the ECFS SOP a commercially available single-use Marquat dual-lumen nasal PD catheter (Marquat Genie Biomedical, Boissy-Saint-Léger, France) (Diameter 2.5mm; Length 80cm) has been used for all NPDs in the Multidose Trial and projects for this thesis (Figure 3.7).

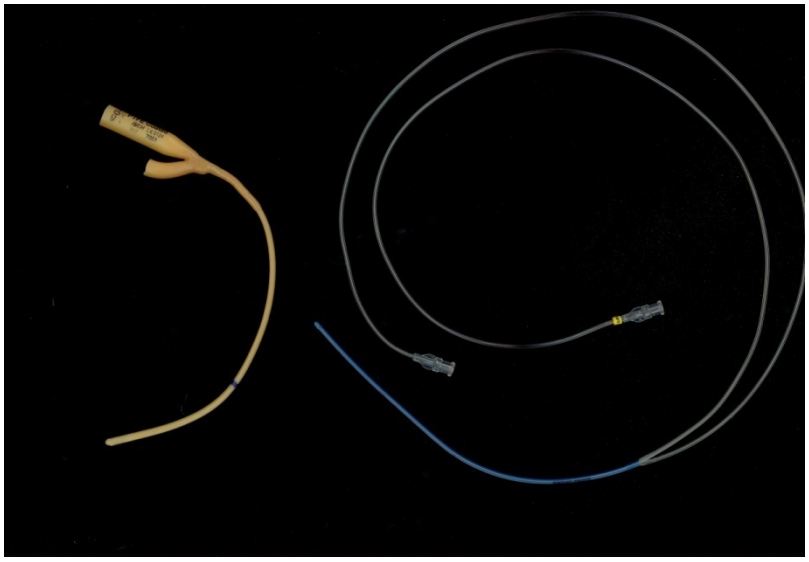


Figure 3.7. Previous NPD techniques used a modified urinary catheter (LEFT); current SOPs recommend the Marquat nasal PD catheter (RIGHT)

3.2.2.2 The 'Recording' Channel

The 'recording' (mucosal) channel is filled with a highly conductive cream, SignaCreme (Henley Medical Supplies, Hertfordshire, U.K.), by using a 2 ml Luer-lock syringe. It is imperative to ensure the entire channel is full and that no bubbles are present in the column of cream, as these disrupt the conductive circuit and may produce a dampened or erratic PD reading. A 5 ml syringe, half-filled with SignaCreme, is introduced at the distal end of the catheter and an external or 'exploring' Silver-Silver Chloride Electrode (Ag/AgCl) (Biosense Medical, Chelmsford, U.K.) inserted into the reservoir of cream and fixed with adhesive tape.

3.2.2.3 The 'perfusing' channel

The catheter's second channel, the 'perfusing' channel, allows ionic solutions to be instilled onto the nasal epithelium and is connected to a perfusion circuit (section 3.2.6). At the proximal end of the catheter are two small openings, approx 0.5 cm from the catheter tip, with the recording orifice making contact with the nasal epithelium and measuring PD, and the other allowing simultaneous perfusion of solutions.

3.2.3 Skin reference electrode

A second Ag/AgCl electrode, the reference (serosal or 'skin') electrode, records the submucosal potential. It has been demonstrated that submucosal space of the skin is isoelectric with that of the airway and can thus be used for this purpose [205]. As this electrode comes into contact with human tissue fluid and cannot be sterilised, it is single use.

A painless, superficial dermal abrasion is made on the forearm of the patient to remove the epidermis and ensure contact with the submucosal layer. A section of the dorsal surface of the forearm is made sterile with a Clinell (70% alcohol; 2% chlorhexidine) skin disinfection wipe (GAMA Healthcare, London, U.K.) and a single-use sterile diamond-tipped dental burr (Henry Schein Dental (U.K.), Gillingham, U.K.) connected to a modified battery-powered hand-fan. The outer dermal layer is removed, leaving a small (<3 mm) sheen on the forearm over which the reference electrode is placed and fixed with Leukoplast Sleek adhesive tape (BSN Medical, Hull, U.K.). To maximise the electrical contact between skin and electrode, the well underneath the electrode is filled with SignaCreme by injecting through the tape from a pre-filled 2 ml syringe with an 18 G needle, sheathed by a sterile, yellow (200 mcl) pipette tip to prevent intradermal penetration.

3.2.4 Recording Potential Difference

Both the recording and reference electrodes are connected to a high-impedance, low-resistance LR-4 millivoltmeter (Logan Research Ltd, Rochester, U.K.), with the recording electrode inserted into the positive (red) port and the reference (skin) electrode inserted into the negative (black) port. This millivoltmeter is connected via a USB into a laptop with Logan Research PD Software (Logan Research Ltd, Rochester, U.K.) installed.

3.2.4.1 Electrical Circuit Offset

The offset in the circuit, created between the two Ag/AgCl electrode placed in 20 ml universal container containing SignaCreme, is measured using the Logan software and accepted if ±5 mV; in instances where the electrode offset is outside these parameters, an alternative combination of electrodes was used. Once measured and accepted, the offset was zeroed on the Logan software programme and the offset value recorded.

The offset is further measured and corrected through the catheter, by placing the proximal end of the catheter with the skin electrode in the universal container filled with SignaCreme. Any large (>1 mV) deviations from the zeroed value, lead to the catheter being pre-primed with fresh SignaCreme to ensure entire filling of the lumen and absence of bubbles. At the end of the NPD, the offset between the electrodes would be repeated to ensure no significant drift had occurred.

3.2.5 Measuring skin potential

Skin potential was measured before every NPD or LAPD to confirm the integrity of the electrical circuit and to confirm that that good skin contact has been made.

With both perfusing and recording circuits setup and both electrodes correctly placed, and with the millivoltmeter turned on and the laptop PD software capturing and recording measurements, the catheter is placed onto the index finger of the patient (with a few drops of SignaCreme).

Skin measurements are typically between -30 and -60 mV (values being more hyperpolarised in the CF than in the non-CF population); a measurement more negative than -30 mV was required to confirm circuit integrity, otherwise the circuit and skin contact were reviewed.

3.2.6 Preparation of the perfusion circuit

50 ml syringes, each containing a different ionic solution, were connected in parallel via a T-piece and 3-way tap to a single extension tubing with a Luer-lock attachment; this in turn was connected to the proximal end of the catheter's perfusing channel and primed with solution (Ringer's solution) ready for use.

A standard NPD protocol uses four syringes/solutions; variations required for individual PhD-specific projects are individually highlighted in each section. Each syringe was mounted on an Alaris® CC Plus with Guardrails syringe pump (CareFusion, CA, USA), with a delivery rate set at 240 ml/hr (4 ml/min); it is important to ensure that the pressure alarm level was set to maximum, as the device will incorrectly detect an occlusion resulting from the pressure delivered at this rate through a small diameter of tubing.

3.2.7 NPD perfusion solutions

3.2.7.1 Manufacture and storage of solutions

All solutions used for the measurement of potential difference (nasal and lower airway) were manufactured by Eastbourne Pharmaceuticals (Eastbourne, U.K.), and were prepared in individual, 50 ml sterile bottles, stored under refrigerated conditions (4 – 8 °C) and had an expiry date of 3 months from date of manufacture. The solutions were removed from refrigeration on the day required and allowed to warm to room temperature prior to use. The batch number and expiry date of all solutions were recorded alongside the recipients' clinical reference details, in keeping with RBH pharmacy traceability policies.

3.2.7.1 Protocol for perfusion of solutions

The following solutions were sequentially perfused at 240 ml/hr through the catheter, and the mucosal response recorded. Once NPD stability had been

achieved, the solution was switched off and the next solution perfused. Whilst variations in the definition of 'stable' exist, a change of <1 mV in 30 seconds was used for all NPDs discussed in this thesis [204].

All the NPDs performed on the Multidose Trial and for this thesis use the following regime, in sequence, unless otherwise stated.

i. Ringer's Solution provides a basal PD and was perfused until there is stability in the PD trace for 30 seconds.

ii. Ringer's Solution with amiloride (0.1 mM) (RA) was perfused for a minimum of 3 minutes and until stability was achieved for 30 seconds.

iii. Zero Chloride Solution with amiloride (0.1 mM) (ZCA) – ZCA was perfused for a fixed time of 5 minutes.

iv. Zero Chloride Solution with Amiloride (0.1 mM) and isoprenaline (10 μM) (ZCAI): owing to the short (2 hr) expiry time of isoprenaline once opened, isoprenaline was added to a ZCA solution immediately prior to use, and was perfused for a fixed time of 5 minutes.

3.2.8 Positioning the nasal catheter

3.2.8.1 Inserting the catheter into the nasal cavity

With the patient's head in a neutral position and sitting face-to-face with the operator, and with the millivoltmeter/laptop measuring data, the catheter was slowly advanced along the floor of one nostril whilst watching the PD response on the monitor in real-time. The exploring catheter had a blue indicator stripe running down its length, meeting with the recording 'orifice' – to ensure consistency in catheter placement. This was kept on the left-hand side of the operator when performing measurements.

3.2.8.2 Choosing the optimal catheter position

The catheter is advanced beyond the first 2 cm from the exterior nares (onto ciliated epithelium) with the operator aiming to identify the site of the highest (most negative) PD measurement; the first time a NPD was performed the operator made an assessment of both nostrils before fixing the catheter in place.

As the catheter had distance markers (at 1 cm intervals) measuring from the distal tip, the operator records the distance from tip to the external nares and the side of nostril. All subsequent NPDs were made at this same location to allow comparison against baseline readings.

3.2.8.3 Fixing the catheter in place

The catheter was fixed in position using 2-3 Steri-Strips™ (3M, Bracknell, U.K.) wrapped around the catheter and adhered onto the nasal bridge; closer attention to the monitor was required to ensure that any movement during fixation did not produce a loss in PD measurement, which would require repositioning of the catheter.

3.2.8.4 Catheter adjustments during the NPD

The catheter remained in position during the entire NPD and was not adjusted except for in exceptional circumstances, such as loosening of the Steri-Strips adhesive from the drippings solutions or where mucus blocks the catheter tip and electrical contact was lost. Under these circumstances, the catheter was re-fixed with more adhesive, or flushed with electrode cream and reinserted.

3.2.9 Positioning the patient during the NPD

Once the catheter was fixed, the patient slightly tilted their head downwards to allow the perfused solution to run out of the nostril and collect in a disposable papier-mâché bowl underneath. By resting the base of their hands on their zygomatic processes and resting their elbows on a table, the patient is able to maintain a still position for the duration of the test. As an alternative, patients could opt to use a head and chin rest device, attached onto the table, and the head position adjusted to suit comfort; this was used in a trivial number of NPDs described in this thesis owing to the patient preference.

During the test, it was important for patients to remain still, quiet and maintain a gentle breathing pattern avoiding sniffing and nasal breathing which would alter the catheter position and, hence, the PD measurement.

3.2.10 Recording of PD data

To make an electronic mark on the PD trace at the moment of change between each solution being perfused, the 'operating' button on the millivoltmeter was momentarily flicked (from 'O/P' (operating) to 'CAL' (calibration), and back), producing a positive deflection on the PD trace.

Once the NPD was completed, the recording was stopped and the trace saved according using a patient specific code; the trace was also 'data-dumped', producing a data-text file to allow the PD to be imported into Microsoft Excel for analysis.

Data were also recorded on paper in case any electronic data was inadvertently lost. Any relevant events occurring during the NPD, for example significant coughing, power failure, syringe driver occlusion, were also recorded on the paper worksheet.

3.2.11 Examples of nasal PD traces from healthy and CF individuals

An example of a typical NPD trace from a healthy (non-CF) control and a person with CF are shown in Figure 3.8. These demonstrate the significant electrophysiological differences between the two populations, namely the difference in basal PD, the magnitude of response to amiloride and the presence/absence of a PD response to stimulation of chloride transport.

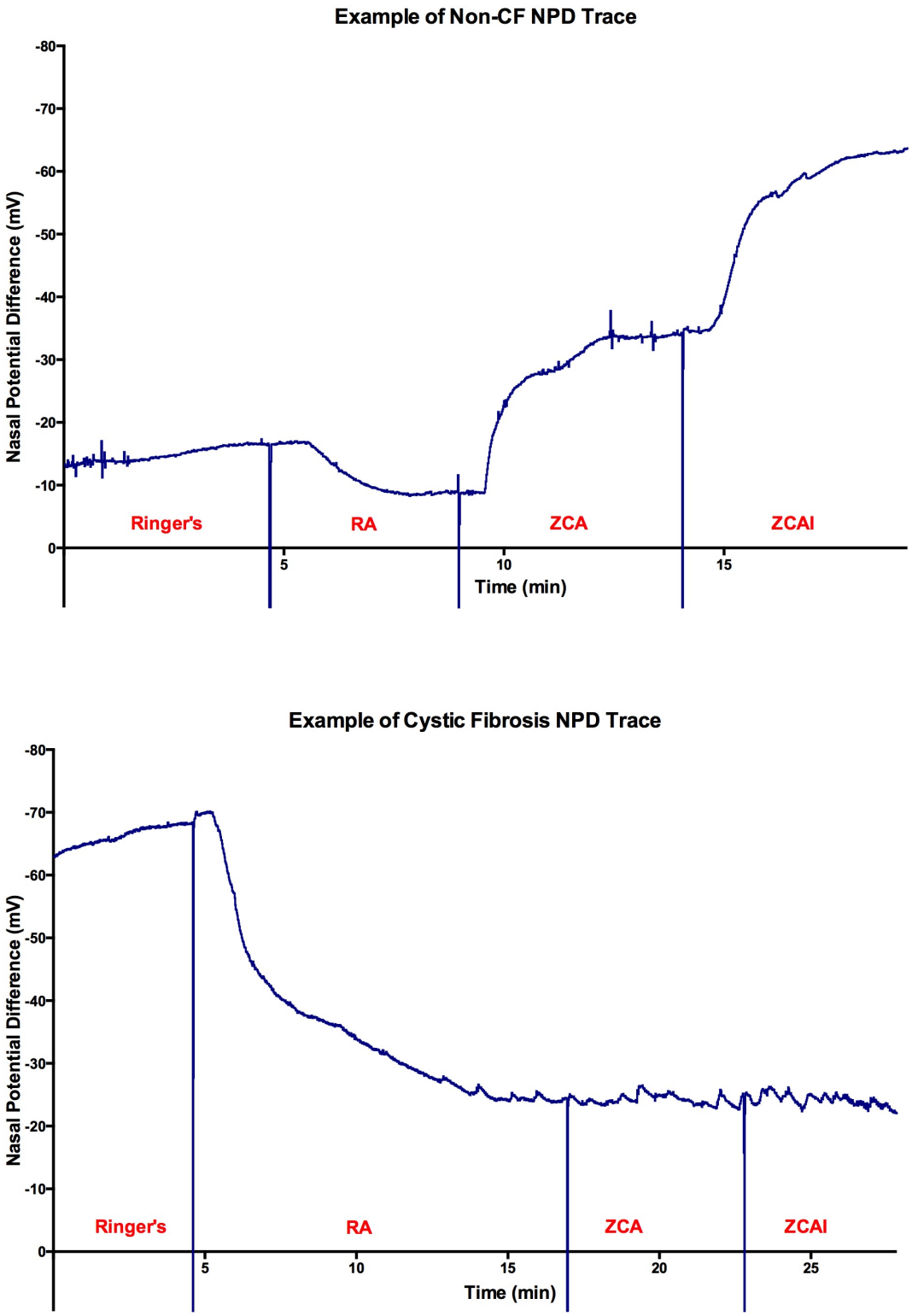


Figure 3.8. Example of nasal potential difference traces from a non-CF volunteer (TOP) and a CF patient (BOTTOM). Phases of the NPD perfusion are labelled: Basal (Ringer's), RA (Ringer's/Amiloride), ZCA (Zero chloride/amiloride) and ZCAI (zero chloride/amiloride + isoprenaline).

3.2.12 Performing blinded NPDs

NPDs were performed 'on treatment' during the MDT. Since previous gene therapy trials had demonstrated a chloride response following active treatment, a similar response in the MDT could have been obvious to the NPD operator and risk unblinding. This was particularly relevant as the majority of NPDs were performed by MDW and KH, who were also responsible for the clinical assessment and recording of adverse events in the same trial patients. It was therefore imperative to maintain blinding during the trial, whilst still performing NPD.

To overcome this risk, it was decided to perform all on-treatment NPDs 'blinded'. This involved a second ('unblinded') operator being 'the eyes' of the NPD trace and the procedure was performed (by MDW or KH) as a two-person technique.

All second operators were independent of the MDT and would therefore be unaffected by any unblinding by NPD responses. Throughout the duration of the trial, 4 additional operators were chosen from administrative staff in the Department of Gene Therapy. Each was trained by MDW or KH to understand the methods and pitfalls of NPD. It was imperative for both the principal (blinded) and second operators to discuss the stability of the trace, identify the appropriate time to change NPD solutions, and to report any loss of contact or other potential problems. Post-hoc validation of this 2-person technique was performed and is discussed in Chapter 4, section 4.6.

3.3 Lower Airway Potential Difference Measurement

Measurements of lower airway potential difference (LAPD) are made via bronchoscopy and follow similar principles and techniques as those described with NPD. However, during bronchoscopy, a single-lumen catheter was utilised for both perfusion of solutions and recording of PD. The method was originally

described by Knowles *et al* [206] and further used by Alton *et al* [207]; LAPD has been validated in CF, demonstrating differences to non-CF individuals [208], and has served as a measure of efficacy in the gene therapy Pilot Study [176]. A pre-existing SOP from the Department of Gene Therapy was used for the MDT and followed by all members of the team involved with LAPD measurement.

A photograph of LAPD measurements being taken during the MDT is shown in Figure 3.9, with a schematic diagram explaining an overview of LAPD set-up in Figure 3.10.

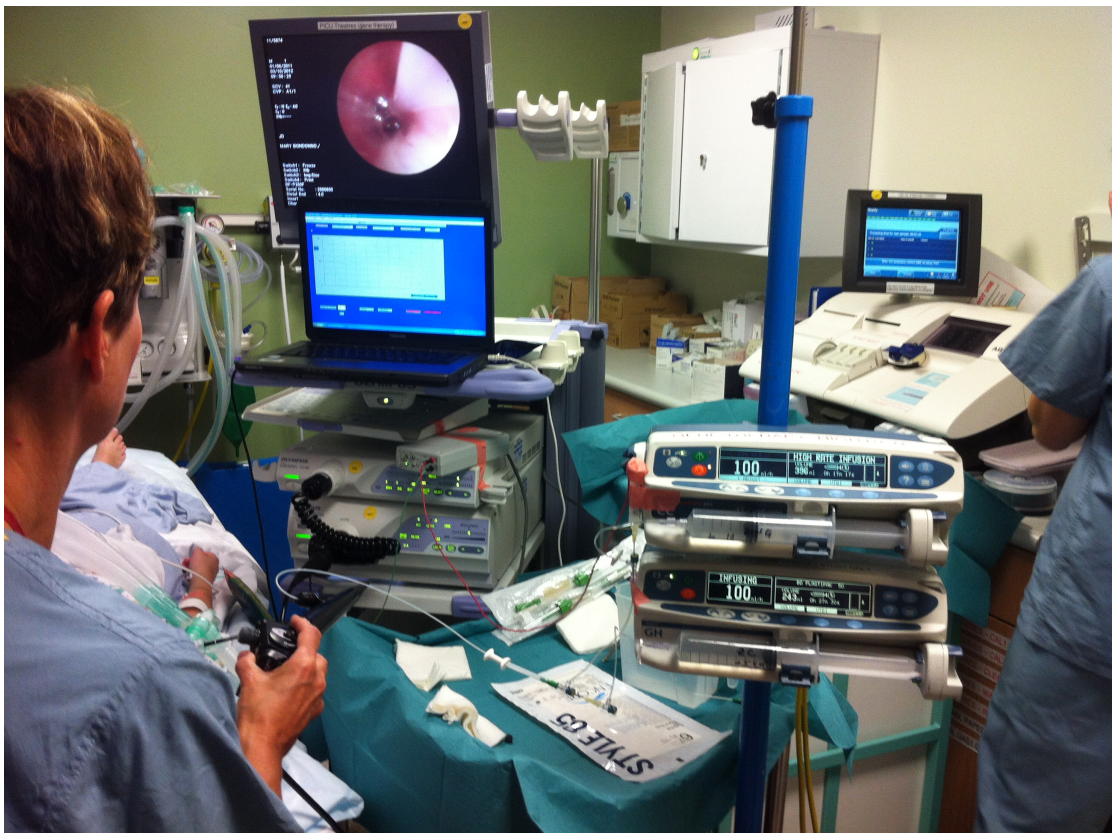


Figure 3.9. Photograph of a patient having LAPD measurements taken (via bronchoscopy) in the Multidose Trial. The image on the TV monitor shows a 5th generation bronchus where peripheral measurements are taken.

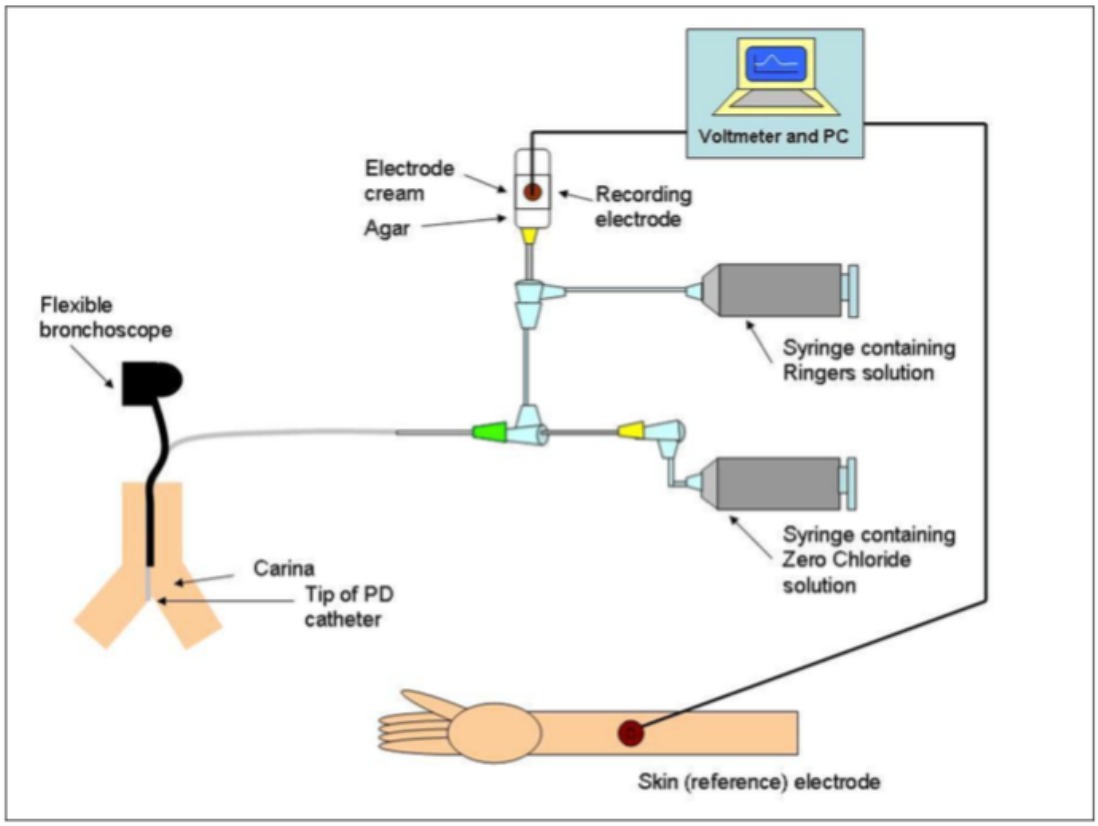


Figure 3.10. Schematic diagram representing the LAPD set-up, using a single-lumen catheter (section 3.3.3) (image courtesy of Dr Gwyneth Davies).

3.3.1 Single-lumen perfusing/recording catheter

The diameter of a single operating channel within the bronchoscope limits the size of the LAPD catheter used and therefore, in contrast to NPD, a single-lumen catheter is used. A sterile, plastic sheath from around a bronchial cytology brush (the wire brush being discarded) (Olympus, Southend-on-Sea, U.K.) is adapted for the purpose. As this style of catheter possesses only one lumen, the channel is used for both perfusing and recording using the continuous stream of ionic solutions as the conducting medium.

3.3.2 LAPD recording circuit

Prior to use, both electrodes were checked to ensure they had an offset ±5 mV.

To record the LAPD via the column of perusing solution, an 18 G Abbocath-T™ is inserted into the airway catheter, which interfaces with an agar-filled syringe that bridges the conduction to the recording electrode. The agar syringe is prepared in advance by warming 4% agar powder in 3 M KCL thus allowing it to dissolve, and pouring at least 1 ml of this solution into a 5 ml syringe (with the plunger removed) and allowing to solidify overnight. When used, the agar syringe is topped with SignaCrème where the recording electrode is inserted and fixed with adhesive tape. A further electrode offset was corrected through the catheter, to identify any problems with the measuring circuit prior to use with a patient.

The external electrode is attached to a skin abrasion in an identical fashion to that previously described. Both electrodes are again inserted into a millivoltmeter connected to a laptop running Logan PD software.

3.3.3 Set-up of the perfusing circuit

As the perfusing system also records the PD, it is imperative to minimise any bubbles within the circuit, as they will interrupt the column of solution 'reading' the PD and therefore disrupt the measurement. In addition to close attention to detail and flushing air from the circuit during set-up, warming of PD solutions overnight to 37 °C and allowing them to cool back to room temperature had been noted during the Pilot Study to reduce micro-bubble formation and thus was adopted.

Each solution is carefully aspirated into a 50 ml syringe (avoiding entraining air bubbles) and connected through an interlocking system of rubber-ended T-

pieces and 19 G needles, via a common central column, to both the agar-syringe and perfusing system.

Two solutions are used, both perfused at 100 ml/hr:

i. Ringer's Solution – to provide a basal measurement at proximal and distal airway sites, perfused until the LAPD is stable;

ii. Zero Chloride + isoprenaline (10 μM) solution (ZCI) (without amiloride) – perfused at the distal (5th generation) lower airway for 5 minutes to measure the combined passive and stimulated chloride secretion.

3.3.4 Measurement of lower airway PD

All bronchoscopies were performed under total intravenous general anaesthesia (propofol and remifentanyl) to (a) remove the need for inhaled volatile or topical local anaesthesia that block amiloride-sensitive sodium channel function [209], (b) make it a safer procedure for the patient with regards to control of the airways, and (c) to limit any interference in the recording such as movement and coughing.

The bronchoscope was operated by JCD, with MDW/KH being responsible for the preparation and control of the PD circuit, the perfusion of solutions, and recording of data. Once anaesthesia had been established, a flexible fibreoptic bronchoscope was inserted through an endotracheal tube. After initial inspection of the airways, the PD catheter was inserted through the operating channel of the bronchoscope and LAPD measurements were made by butting the distal end of the catheter against the side of the airway wall and perfusing solutions whilst the catheter remained stationary. It was not uncommon to experience a loss in the PD circuit contact during the change of solution owing to microbubbles in the perfusing recording system. In general, contact could be re-established by temporarily increasing the rate of perfusion to flush the system. If contact could

not be re-established, the PD measurement was abandoned and a separate location of measurement considered.

3.3.4.1 LAPD basal measurements

Basal measurements (in response to Ringer's solution) were made at the proximal lower airway at the carina (at 4 positions – anterior, posterior, left side and right side).

3.3.4.2 LAPD distal measurements

Distal lower airway PD measurements are measured at 5th generation bronchus, reflecting the furthest distance the bronchoscope can be inserted and measurements made under direct observation. All LAPD measurements were made at three different sites on the same side of the lung by perfusing Ringer's solution and then ZCI solution (5 mins); in between each measurement, the catheter was removed from the lungs and flushed with Ringer's solution to eliminate residual ZCI. The Consortium felt that three measurements were an appropriate number to be taken, to allow for repeated LAPD meaurements to reduced variability between measurments, without significantly prolonging the anaesthetic time for the patient.

3.3.5 Examples of lower airway PD traces

3.3.5.1 Lower airway basal PD from healthy and CF individuals

Basal PD measurements were taken from the proximal airway at the carina, and at the peripheral airways (5th generation bronchiole). Measurements in children have shown (i) a higher proximal basal PD in CF compared to non-CF children (statistically different at the trachea), and (ii) a progressive fall in basal PD measurements travelling distally through the bronchial tree (Figure 3.11). This

finding was also mirrored in adults with CF, and in comparison to healthy controls [208].

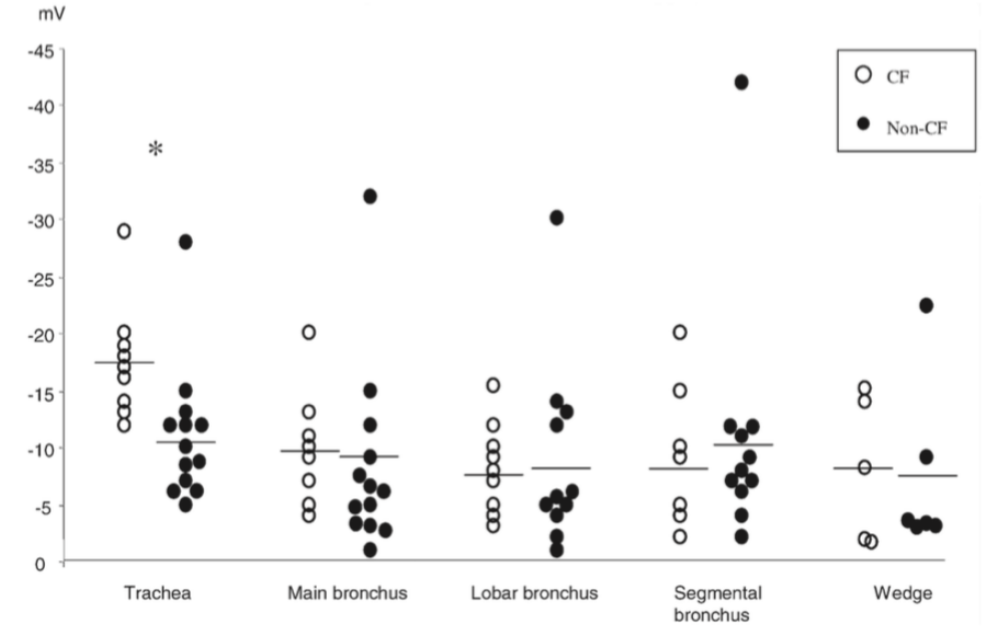


Figure 3.11. Lower Airway Basal PD Measurements in children with CF and without CF (non-CF) (*p<0.001). Reproduced from Davies *et al* [208].

3.3.5.2 Lower airway chloride secretion healthy and CF individuals

Chloride efflux, through CFTR, is measured in the peripheral airways (5th generation bronchus), with an isoelectric (flat) response being seen in CF, but epithelial hyperpolarisation occurs in non-CF individuals. A statistically significant difference in chloride efflux after 3 min of ZCI perfusion at a 3rd generation airway has been demonstrated in children with and without CF (Figure 3.12).

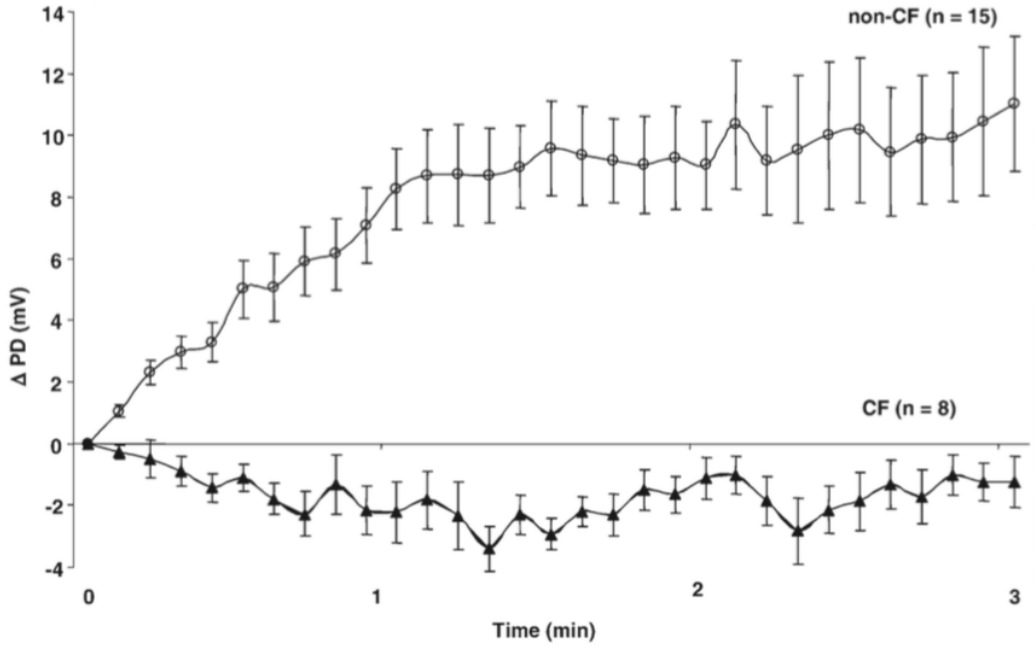


Figure 3.12. Lower airway PD measurement at a 3rd generation airway of chloride efflux in response to a ZCI solution in children with CF and without CF, the difference after 3 min being statistically significant ($p<0.001$).

3.3.6 Airway microbiological samples

Airway secretions were aspirated for standard CF microbiological cultures, with an additional sample sent for alcohol-acid fast bacilli where patients had been unable to expectorate during the clinical visit.

3.3.7 Patient safety and monitoring

Throughout anaesthesia, all patients had their vital signs continuously monitored (HR, BP, SpO_2, RR and end-tidal CO_2), in addition to their ventilation parameters.

Following reversal of anaesthesia and extubation, two members of the research team (at least one being either a registered nurse or doctor) recovered the

patients in the CRF for a minimum of 4 hours. Vital signs (HR, BP, SpO_2, RR and body temperature) were measured regularly, and patients were clinically assessed after 4 hours by MDW/KH prior to discharge home. Each patient's clinical CF teams were notified of the outcome of the procedure prior to discharge in case of any concerns or untoward events.

3.3.8 Prophylactic IV antibiotics

To reduce the risk of a bronchoscopy-induced pulmonary exacerbation or airway-induced bacteraemia, all patients received a single-dose of two intravenous antibiotics, targeted at their sputum cultures and sensitivities, prior to the end of the bronchoscopy (after collecting a lower airway sample for microbiology).

3.4 Collection of Epithelial Cells for Transgene cDNA and mRNA

Epithelial samples were taken from both nasal and lower airway mucosa pre-dosing and at follow-up by brushing the surface with a cytology brush (Olympus, Southend-on-Sea, UK), and processed by the UKCFGTC at Oxford University. The author took all nasal brushing samples on the study, and assisted in the collection of bronchial epithelial samples.

The following describes the method used to obtain these specimens.

3.4.1 Nasal brushing to obtain epithelial cell samples

3.4.1.1 Learning to perform nasal brushing

Nasal brushing is a technique to obtain nasal epithelial cells. All subjects enrolled in the nasal group underwent nasal brushing pre -dosing and at follow-up; most samples were collected by the author. Prior to commencing the MDT, the author

was trained in the technique of nasal brushing by JCD and practiced on at least 12 healthy (non-CF) volunteers. A cell count was performed on each sample, to confirm a sufficient quantity of cells for performing molecular assays.

3.4.1.2 Performing nasal brushing

Nasal brushing samples were taken from patients in the nasal subgroup from both nostrils at a pre-dosing visit (following the last NPD to prevent disruption to the nasal epithelium that would alter the NPD response) and at the final follow-up visit.

Patients were prepared by blowing their nose and were positioned sitting against a wall with their head reclined slightly backwards. Whilst the procedure is uncomfortable and may occasionally cause minor epistaxis, it is safe and any epistaxis short-lived.

Nasal brushing is performed under direct vision; to increase visualisation of the nasal cavity the operator wears a head torch. A sterilised nasal speculum was used to open up the nasal orifice allowing visualisation of the nasal cavity and the inferior turbinate identified. A modified 3 mm interdental brush (Dent-o-care, London, U.K.) was moistened with phosphate-buffered saline (PBS), and then inserted under direct vision beneath the inferior nasal turbinate and then moved anteriorly/posteriorly and rotated for 3-5 seconds.

3.4.1.3 Collection of nasal epithelial cells

The cells were removed by repeatedly passing the brush through a widened yellow pipette tip (1-2 mm of the tip cut off) into an Eppendorf containing 800 µl of cold (4˚C) PBS, and the sample then placed on ice.

The procedure was performed on both nostrils and the samples combined into one tube.

3.4.2 Obtaining an endobronchial epithelial sample

Bronchial wall brushing was performed at the pre- and post-dosing bronchoscopies, with samples being taken from each patient at each visit. After all LAPD measurements were taken, endobronchial cytology brushings and histological biopsies were then taken by JCD from the same lung. A sterile, single-use endobronchial brush (Olympus, Southend-on-Sea, U.K.) was agitated against the epithelium of a 5th generation airway and the bronchoscope removed without re-sheathing the brush, to prevent any loss of cells occurring during re-sheathing of the cytology brush. The brush was agitated through a widened yellow pipette tip directly into an Eppendorf containing cold (4˚C) PBS, and the sample then placed on ice; this was repeated from different locations within the same lung to provide a total of 10 specimens, to increase the overall yield of cells.

3.4.3 Quantification of Transgene cDNA and mRNA

The nasal and bronchial cell samples were each divided into two aliquots, for: (i) total cell count under light microscopy (performed by the Consortium's research technicians in London); (ii) mRNA and DNA extraction for quantification.

DNA and RNA were simultaneously isolated from cells collected by nasal and endobronchial brushing using AllPrep (QIAGEN, Manchester, U.K.), then their levels measured using TaqMan® (Life Technologies, Paisley, U.K.), a real-time assay using polymerase chain reaction (PCR) (with reverse transcriptase where appropriate), designed and optimised to detect and quantify endogenous and plasmid (pGM169)-specific DNA and mRNA from patient cell samples. The TaqMan® assay has been previously used by the Consortium in Oxford to quantify transgene expression from vector-derived mRNA after the administration of DNA/liposome complexes to murine airways [210]. Absolute pGM169 DNA levels were measured and normalised to total genomic DNA; levels of plasmid-derived cDNA were normalised to endogenous hCFTR mRNA. Data

were calculated as the percentage of pGM169-specific to endogenous CFTR copy number.

Whilst the author took or assisted in taking airway epithelial samples, he did not perform any molecular testing – these were performed at the end of the study by the Consortium's research team in Oxford University.

3.5 Statistical Analyses

Data were collated in Microsoft Excel and analysed in GraphPad Prism (version 6). Normality was tested in Prism using the D'Agostino-Pearson omnibus normality test. Relationships between continuous and rank-ordered variables were assessed using correlation coefficients (Pearson and Spearman rank). Comparisons between two groups were made using the paired t-test, a Wilcoxon paired rank tests, or a Mann-Whitney U test (non-parametric data). A Fisher's exact test was used for comparing two groups with two outcomes. Within this thesis, a p value <0.05 was considered significant. Prism was used to create all graphical representation of the data.

Chapter 4: Nasal Potential Difference Measurement

This chapter will focus on the nasal subgroup within the Multidose Trial. It will predominantly be concerned with NPD, and will describe the protocol for the nasal subgroup in the MDT, report electrophysiological outcomes at baseline and in response to gene therapy, and assess correlations between NPD and measurements of lung function. It will also include *CFTR* molecular outcomes from the nasal epithelium. The chapter will finally present a novel method for measuring NPD using a two-person, 'blinded' technique. The methods used within this chapter have been described in chapter 3.

The hypothesis of this chapter is that non-viral *CFTR* gene therapy, instilled at monthly intervals onto the nasal mucosa, will (i) demonstrate electrophysiological changes of *de novo* CFTR function, and (ii) that correlations will exist between nasal PD measurements of ion transport and measurements of lung function.

4.1 Multidose Trial – Nasal Subgroup

The aim was to enrol 24 patients into the nasal group, with a randomisation in favour of gene therapy (active : placebo = 2:1). To be eligible for the subgroup, patients could not have any clinically significant rhinosinusitis or nasal polyposis where the nasal epithelium would be inflamed or abnormal (exclusion criteria Chapter 2, section 2.3.5), or have an exaggerated mean total chloride response (>5 mV). However, the latter was only possible to exclude once NPD had been performed.

4.1.1 NPD Protocol

NPD was performed as per the methods described in chapter 3. The trial protocol stated that patients would undergo 3 pre-treatment NPD (baseline) measurements, a variable number of on-treatment NPD measurements (optional), and 2 follow-up NPD measurements (14 ±2 and 28 ±2 days following dose 12) according to the following protocol (Figure 4.1).

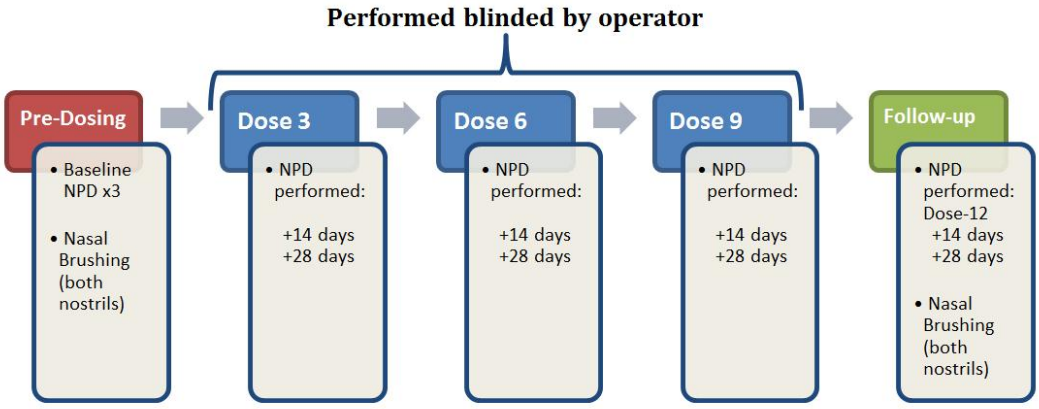


Figure 4.1. Flowchart representing the protocol for the nasal subgroup enrolled into the Multidose Trial.

4.1.2 'On treatment' NPDs

All patients were invited to have NPD measurements taken at 14 ±2 days and 28 ±5 days after receiving 3, 6, and 9 doses of treatment. These measurements were performed blinded with 2 operators (Chapter 3, section 3.2.12).

As the 28-day NPD coincided with the subsequent dosing visit, these were more likely to be performed unless the patient was clinically unwell or unable to attend the visit. Logistically, for patients to attend the CRF at 14-days post-dosing, it was more burdensome, owing to patients' work commitments and the distance of travel, and so was performed in a minority of patients (on 6 occasions in 4 patients). Owing to the difficulty in obtaining 14-day measurements, this time interval was abandoned during the study and was not used in final data analysis.

4.1.3 Analysis of NPD traces

The data files from all NPD traces were loaded onto a PC and file names changed using an online randomisation tool to blind the data for analysis. The traces were blinded to treatment arm, and to time-point of the measurement within the

study (i.e. pre-treatment, on treatment, or follow-up). Blinding and renaming of all filenames was done by KH.

Each NPD trace was imported in Microsoft Excel where it was converted from a data text file into a numerical Excel file, and a graphical trace drawn. All blinded traces were independently analysed by MDW and JCD in Excel calculating the PD values electronically over an approximate 15-20 sec period of stability prior to changeover to the next phase. The individual NPD scores and the agreement between each observer were compared, with a predefined level of agreement of ≤1 mV between each absolute and delta value, including tCl^-. Each value exceeding this threshold was reviewed again by both observers and modifications to their score were made as judged appropriate. To ensure that traces were appropriately interpreted and scored by both MDW and JCD, a further analysis of the traces was made by an independent consultant (Steve Smith) using printed versions of the NPD traces (baseline and follow-up only). These scores were compared against the final data agreed by MDW and JCD. For any traces where no consensus could be obtained, or where the results differed from those reported by Steve Smith, or when traces were difficult to interpret, a final moderation of the data was made by EA using printed traces

4.1.3.1 NPD trace indices

Every trace was 'scored' for the following absolute and change (delta value (d)) in PD for the following indices:

- Basal PD – in response to a perfusion with Ringer's solution
- Amiloride / dAMIL – in response to perfusion with RA solution
- ZCA / dZCA – in response to perfusion with a zero chloride + amiloride solution for 5 min

- ZCAI / dZCAI – in response to perfusion of a zero chloride + amiloride + isoprenaline solution for 5 min

- Total chloride response (tCl^-) – the sum of the dZCA + dZCAI phases.

The time for a perfusing solution to leave the syringe and travel to the tip of the nasal catheter was measured as 30 sec (data not presented within this thesis). All delta (d) 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.

4.1.3.2 Defining a chloride response

Whilst NPD is used clinically to exclude CF based on an accepted diagnostic threshold of a total chloride response < -5 mV, the clinical relevance of the same degree of chloride response is unknown when used as a trial outcome measure of CFTR function [204]. There is no standardised definition of a chloride 'response' to assess changes in CFTR efficacy, and assessments of changes from pre-treatment to post-treatment, or comparisons to an untreated population may be used. For the MDT, changes in a non-CF direction were deemed clinically meaningful and will be reported.

Previous work for a PhD Thesis by Dr Gwyneth Davies [211] applied three methods to NPD results following a single dose of gene therapy (Pilot Study) to assess efficacy of gene transfer. The methods applied to the results of the delta-chloride phase were as follows:

1. Any follow-up value was greater than any pre-test value;
2. The change between the mean pre- and post-dose test was >5 mV;
3. The absolute total chloride secretion was > 5mV.

The author elected to use these parameters as one method to assess the response of the follow-up data as a supplementary analysis to the MDT results;

he furthermore chose to make additional calculations by applying published NPD equations, used clinically to help distinguish CF NPD traces from non-CF. The equations used were:

i. Wilschanski equation: $e^{(\text{total chloride response}/\ \text{response to amiloride})}$ (values >0.70 are supportive of CF) [212]

ii. Sermet equation: -0.11 Δtotal chloride response – 0.05 dAMIL (values CF≤0.27 are supportive of CF) [213].

4.2 Results: Nasal Subgroup, Trace Analysis and Baseline NPDs

4.2.1 Nasal Subgroup Enrolment

A total of 36 patients enrolled into the nasal subgroup, with 12 patients exiting the nasal group prior to dosing: 7 patients failed screening for the MDT for other reasons (Chapter 2, section 2.6.1) and 5 patients were excluded from the nasal subgroup owing to a total chloride secretion exceeding the upper limit of normal for CF (>5 mV) – see section 4.2.6.2. 3 patients withdrew from the study during the dosing phase of the trial – 2 patients each cultured an exclusionary microorganism (*MRSA* and *M. abscessus*, both in the active treatment group), the other patient withdrew his consent (placebo group). A summary of the enrolment, completion and withdrawal of the nasal subgroup is shown in the consort diagragm in Figure 4.2.

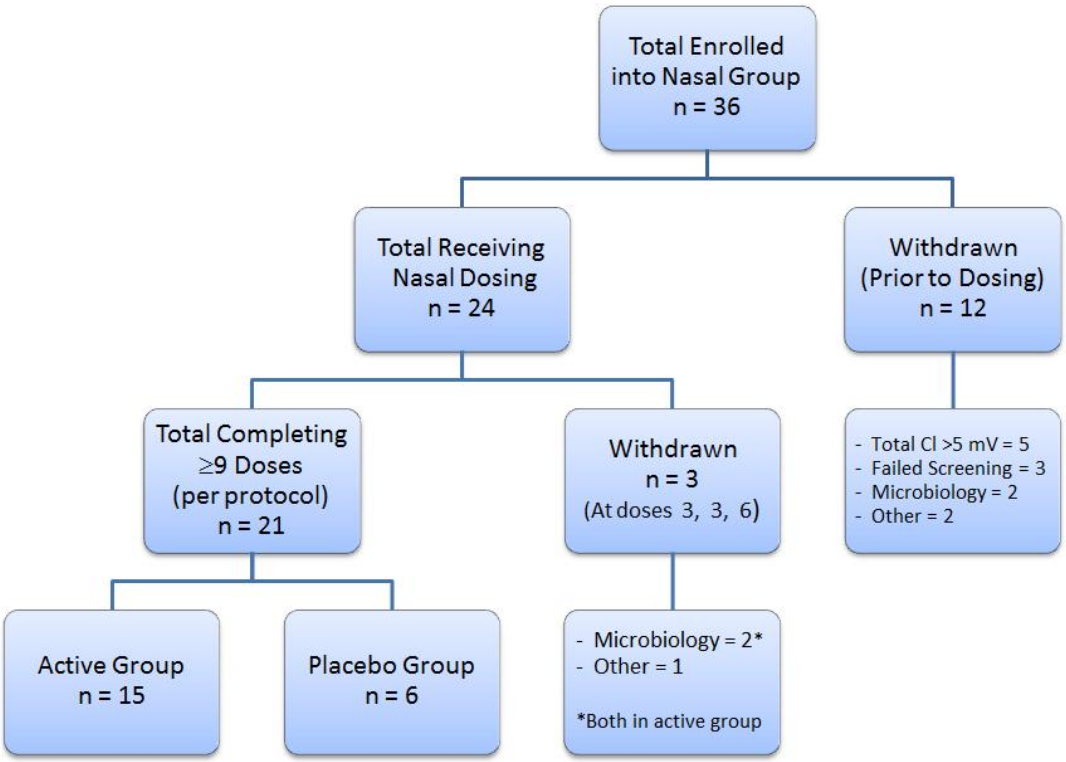


Figure 4.2. Consort diagram of enrolment, withdrawal and completion of the nasal subgroup.

4.2.2 Nasal Subgroup – Baseline Demographics

A total of 21 nasal subgroup patients completed the trial according to per protocol definitions (receiving ≥9 doses of treatment); and the analyses made in response to treatment relate to this population.

For other, cross-sectional analysis requiring only baseline data, for example for the purpose of correlations without treatment, all NPD measurements that were performed have been included to increase data points and will be used throughout this thesis.

All patients had 3 pre-dosing (baseline) NPD measurements performed; all were included in the analysis, except for one patient (LON4032) where 2 of 3 traces were excluded at baseline owing to a flat-NPD response.

The population studied in the nasal cohort was not significantly different in basic demographics and baseline lung function to that of the entire trial population in both placebo and active groups. A summary of the demographics of the "per protocol" (≥9 doses) nasal subgroup population are tabulated in Table 4.1.

	Per Protocol Population (≥ 9 doses) (n=21)	
	Placebo (n=6)	**Active (n=15)**
Sex, n (%)		
Male	4 (67)	7 (47)
Female	2 (33)	8 (53)
Age, yr, mean (SD)	28.7 (7.8)	23.4 (8.6)
ppFEV$_1$, mean (SD) (%)	63.2 (8.3)	65.1 (7.6)
LCI, mean (SD)	11.3 (2.9)	10.8 (2.2)
***CFTR* genotype, n (%)**		
F508del/F508del	4 (67)	8 (53)
F508del/other	2 (33)	7 (47)
Baseline PD, mean (SD) (mV)		
Basal PD	-51.4 (18.3)	-54.4 (14.3)
dAmil	31.1 (19.3)	31.1 (2.6)
tCl$^-$ Response (delta tCl$^-$)	2.4 (3.8)	1.6 (2.7)

Table 4.1. Baseline (pre-dosing) demographics of the nasal subgroup (per protocol population), including genotype, mean baseline lung function and NPD measurements. ppFEV1 – percent predicted FEV_1; tCl^- - total chloride response.

4.2.3 Number of NPD measurements performed

In total, 203 NPD measurements were made throughout the MDT, with MDW performing 105 (52%) of these measurements; 24 measurements were made in patients who were subsequently withdrawn from the nasal subgroup. Figure 4.3 shows the proportion of NPDs performed or omitted at each visit in the per protocol nasal subgroup population (the reducing total of patients reflects the 3

patients withdrawing during dosing); Figure 4.4 summarises the proportion of NPDs performed or omitted in the active and placebo subgroups.

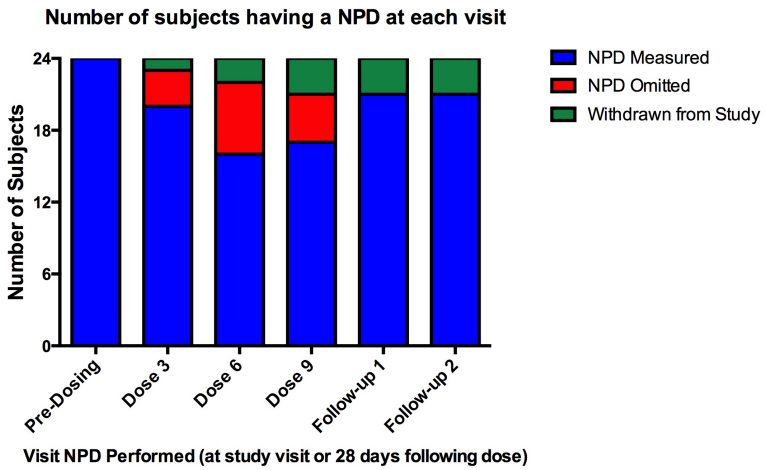


Figure 4.3. Bar chart showing number of NPDs performed or omitted per dosing visit in the per protocol nasal subgroup (n=24).

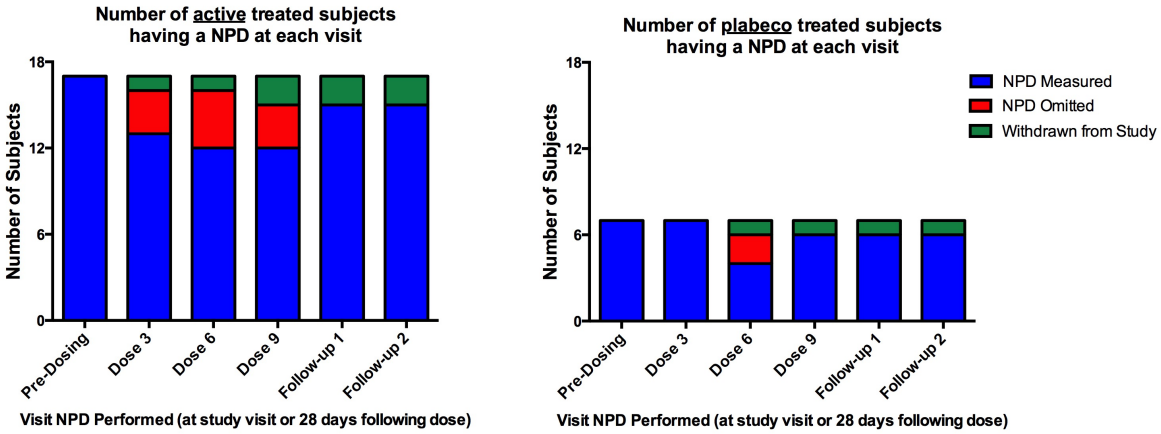


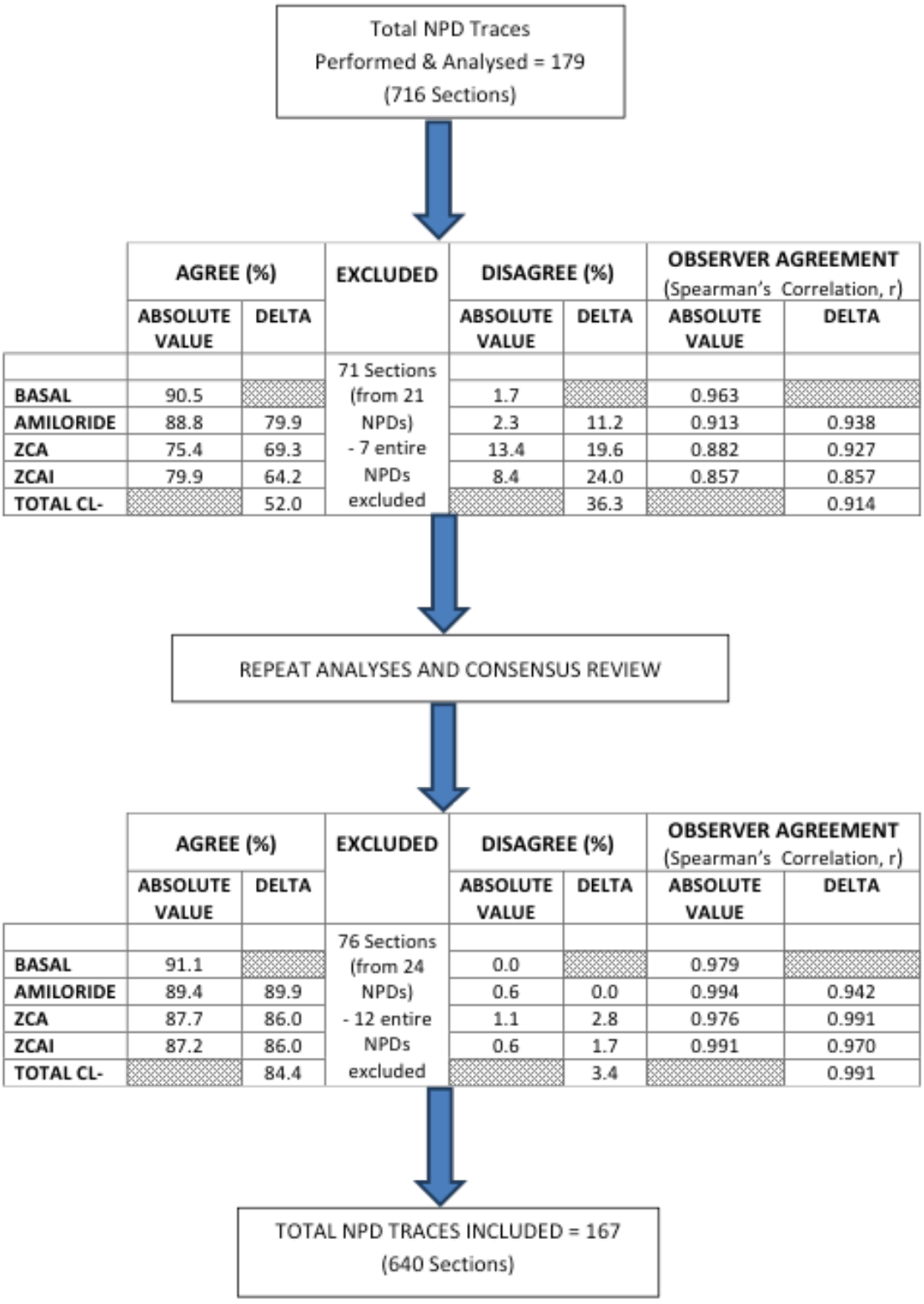
Figure 4.4. Bar chart showing number of NPDs performed or omitted per dosing visit in (i) LEFT: active subgroup (n=17); and (ii) placebo subgroup (n=7).

4.2.4 Analysis of NPD traces

In total, 179 NPDs were analysed, with each phase of the NPD being independently assessed (a total of 1432 NPD 'sections' – either absolute or delta PD values). Agreement between the interpretation and NPD score was made between the observers (MDW and JCD), as outlined in section 4.1.3. For every trace where the measured value or delta was outside the level of agreement (i.e. > 1mV), both scorers reanalysed the trace. This process continued until no further agreement could be made. In total, five analyses of the data were made; any traces where consensus could not be reached were moderated by EA as previously described.

In the first round of analysis, disagreement between the scores of the observers was found in 206 (14.4%) sections – the major proportion of these were in the chloride indices and in part related to a small number of NPD traces having a ZCA and ZCAI phase lasting longer than 5 min, and traces being fractionally outside the <1 mV limit. Following serial adjustments from both observers, the final (fifth) analysis demonstrated a considerable improvement in agreement, with 18 (1.3%) sections remaining where agreement was unattainable – these were referred for moderation. Despite a sizeable (14.4%) disagreement in the first round, the overall inter-observer agreement was good reflecting that all scores assigned were close. The correlation between both scores improved over the course of the analyses, demonstrating that the results were more congruent.

Figure 4.5 shows a summary of the inter-observer agreement and correlation between the first and the final NPD analysis.



	AGREE (%)		EXCLUDED	DISAGREE (%)		OBSERVER AGREEMENT (Spearman's Correlation, r)	
	ABSOLUTE VALUE	DELTA		ABSOLUTE VALUE	DELTA	ABSOLUTE VALUE	DELTA
			71 Sections (from 21 NPDs) - 7 entire NPDs excluded				
BASAL	90.5			1.7		0.963	
AMILORIDE	88.8	79.9		2.3	11.2	0.913	0.938
ZCA	75.4	69.3		13.4	19.6	0.882	0.927
ZCAI	79.9	64.2		8.4	24.0	0.857	0.857
TOTAL CL-		52.0			36.3		0.914

	AGREE (%)		EXCLUDED	DISAGREE (%)		OBSERVER AGREEMENT (Spearman's Correlation, r)	
	ABSOLUTE VALUE	DELTA		ABSOLUTE VALUE	DELTA	ABSOLUTE VALUE	DELTA
			76 Sections (from 24 NPDs) - 12 entire NPDs excluded				
BASAL	91.1			0.0		0.979	
AMILORIDE	89.4	89.9		0.6	0.0	0.994	0.942
ZCA	87.7	86.0		1.1	2.8	0.976	0.991
ZCAI	87.2	86.0		0.6	1.7	0.991	0.970
TOTAL CL-		84.4			3.4		0.991

Figure 4.5. Summary of agreement between scoring of NPDs between MW and JCD, at first analysis and following consensus review at final (5th) analysis.

4.2.5 Basal and amiloride responses at baseline

This section will report the baseline (pre-treatment) results of basal response, and response to amiloride in all patients enrolled into the nasal subgroup and intention to treat population (i.e. having at least one nasal dose), and the variability of the results.

A total of 36 patients were enrolled into the nasal subgroup and had at least one measurement taken (in total 94 NPD measurements were made at baseline, with 80% of patients having 2 or more measurements taken); 24 patients remained in the intention to treat population, each having 3 NPD measurements taken pre-dosing. The basal and dAmil values at baseline for these groups are shown in Table 4.2.

	All Enrolled Patients (n = 36)	**Intention to Treat Population (n = 24)**
Basal PD (mV) Mean (SD)	-57.0 (15.6)	-55.0 (15.6)
dAmil (mV) Mean (SD)	34.6 (20.6)	31.4 (14.2)

Table 4.2. Table summarising the mean (SD) basal and dAmil NPD measurements at baseline for all patients enrolled into the nasal subgroup, and those in the intention to treat nasal subgroup.

4.2.5.1 Coefficient of variation for baseline NPD (basal and dAMIL)

An assessment of the variability of repeated NPD measurements at baseline (pre-treatment) was made. From all patients enrolled into the nasal subgroup (n=36), and having more than one pre-treatment PD measurement performed (n=30), the mean coefficient of variation (*CV*) was calculated for both (i) basal and (ii) dAMIL indices (Table 4.3).

Mean coefficient of variation (%)	All Enrolled Patients having repeated NPDs (n = 30)
Basal PD	13.1
dAMIL	25.9

Table 4.3 – Mean coefficient of variation (CV) for (i) basal, and (ii) dAmil responses in the nasal subgroup of the Multidose Trial for all patients enrolled into the nasal subgroup and having more than 1 measurement taken at baseline.

4.2.5.2 Distribution of individual baseline NPD indices (basal and dAMIL)

From all patients enrolled to the nasal subgroup that had more than 1 NPD measured at baseline, the distribution for variability of their individual (i) basal, and (ii) dAMIL NPD measurements is shown in Figure 4.6 and Figure 4.7 respectively.

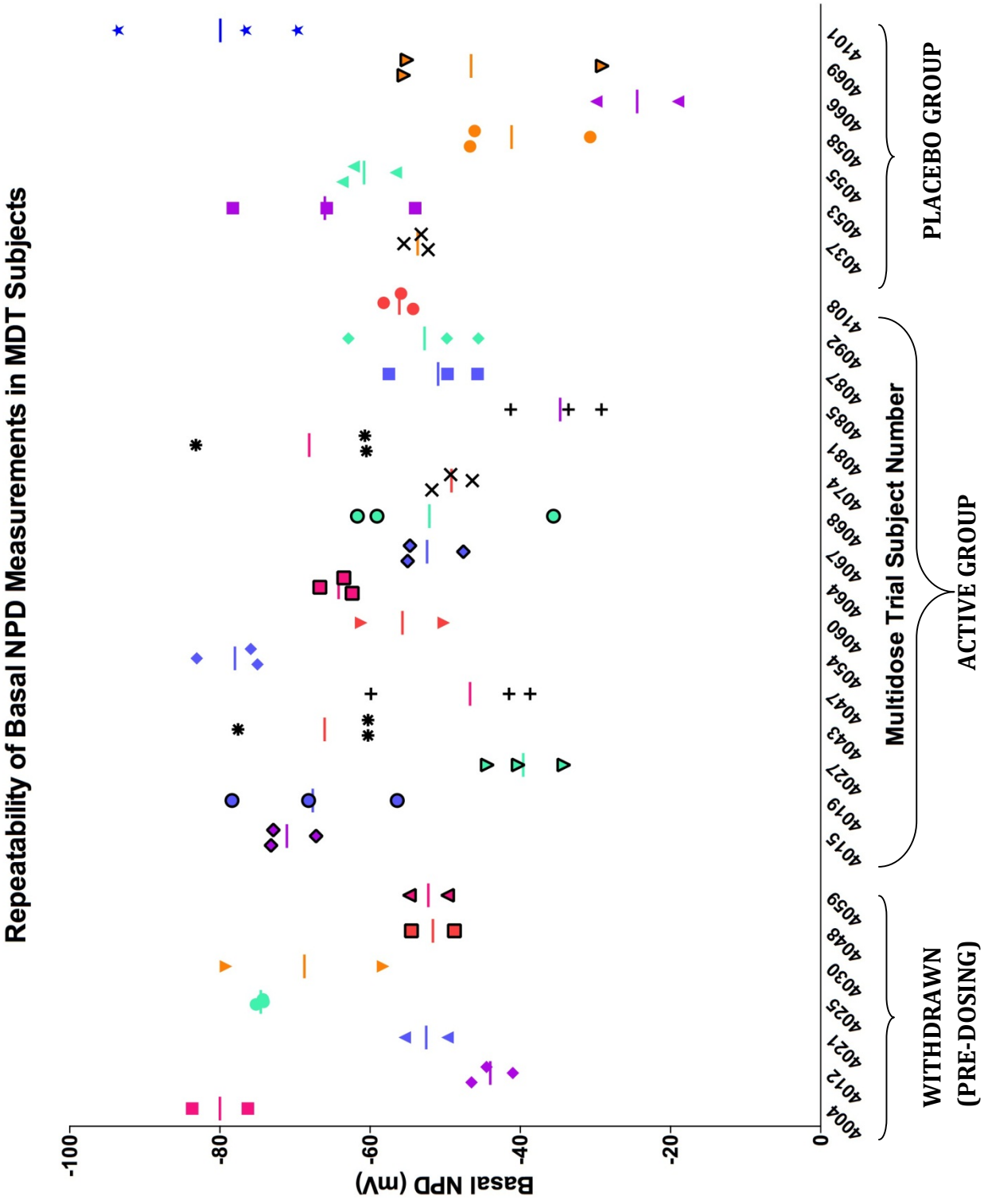


Figure 4.6. Repeatability of basal NPD measurements at baseline in Multidose trial subjects (n=30).

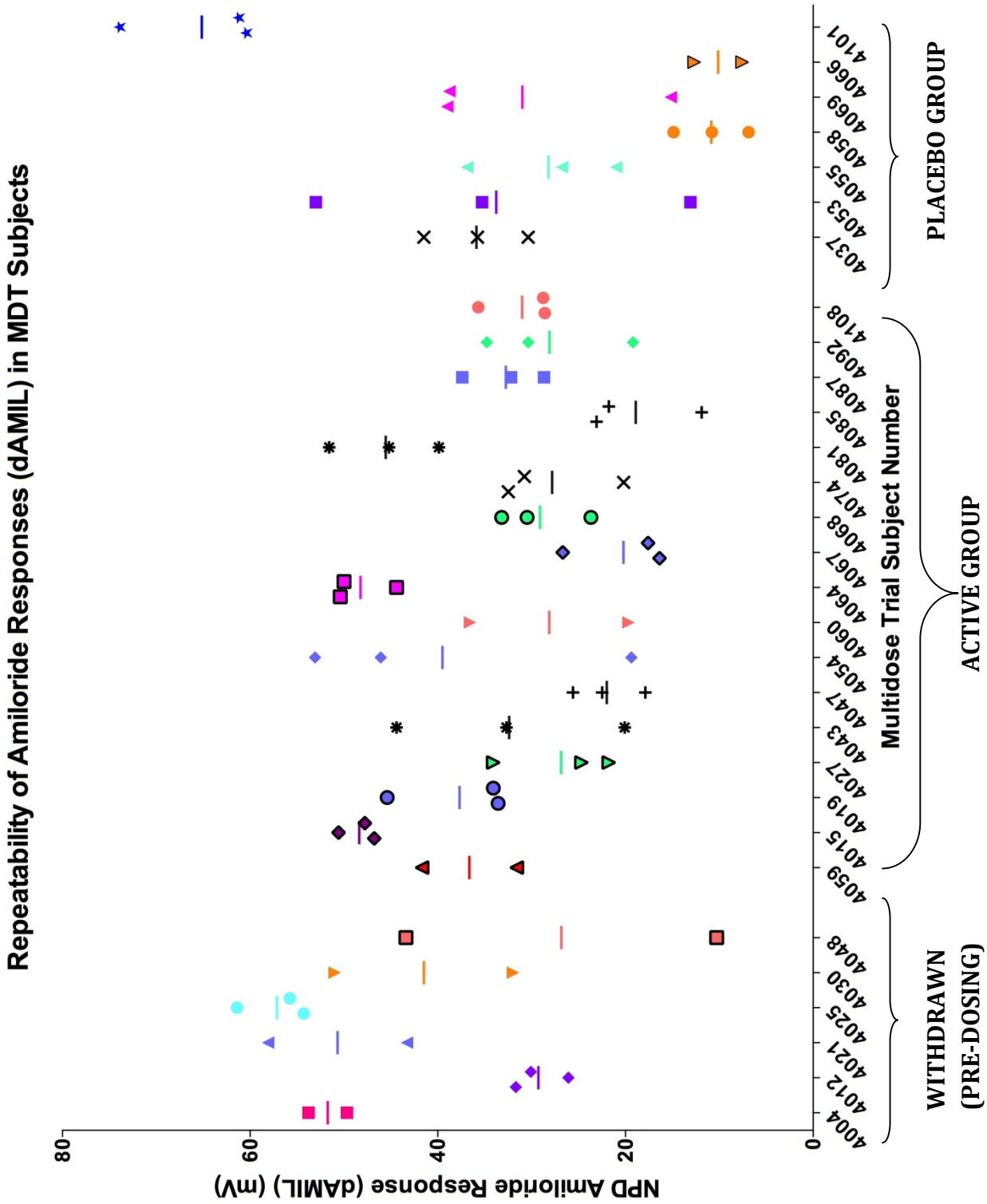


Figure 4.7. Repeatability of dAMIL NPD measurements at baseline in Multidose trial subjects (n=30).

4.2.6 Total chloride response at baseline

The measurement of the NPD in response to chloride-free solutions was measured at baseline in the same 36 enrolled patients, and in 24 patients in the intention to treat nasal subgroup, with the former including 5 patients who were subsequently withdrawn for having an excessive chloride response – the measurements at baseline are summarised in Table 4.4.

	All Enrolled Patients (n = 36)	**Intention to Treat Population (n = 24)**
ZCA (mV) Median (IQR)	-3.0 (-3.0, 3.0)	1.4 (-2.0, 3.6)
ZCAI (mV) Median (IQR)	-0.3 (-1.5, 1.6)	0.1 (-1.0, 1.8)
Total Cl^- (mV) Median (IQR)	1.5 (-3.0, 4.5)	1.8 (-1.8, 5.1)

Table 4.4. Table summarising the median (IQR) NPD chloride responses (ZCA, ZCAI & tCl^-) at baseline for all patients enrolled into the nasal subgroup (including pre-dosing and on treatment withdrawals) and intention to treat nasal subgroup.

4.2.6.1 Variability within subject for chloride measurements

CV is an inappropriate measure for chloride indices in CF patients owing to their mean value being close to zero. To understand the intra- and inter- patient variability for chloride responses, each patient's individual tCl^- response at baseline is shown in Figure 4.8 – this graph has been arranged by patient status (withdrawn pre-dosing, active and placebo treatment group) to demonstrate the similarities in the response of the pre-treated population. This graph emphasises 5 patients who were excluded from the nasal group based on their mean chloride secretion at baseline exceeding the predefined threshold (-5 mV response) – these are discussed in greater detail in section 4.2.6.2. Whilst some patients

demonstrated at least one measurement of at least -5 mV, their mean value was within the acceptable inclusion range.

4.2.6.2 Withdrawal from nasal subgroup for excessive chloride secretion

The recognised upper limit of normal for total chloride secretion is accepted as -5 mV in response to perfusion of the nasal mucosa with ZC and ZCI solutions, each for 5 minutes [204]. For the MDT, the acceptable mean baseline total chloride response (tCl^-) was - 5 mV and patients exhibiting a response greater than this were excluded from the nasal subgroup; they were not however excluded from the trial as a whole nor from the lower airway subgroup, as there are currently no data correlating the two indices.

Five patients were identified as having a mean tCl^- response more negative than -5 mV and were excluded from the subgroup before dosing; one of these patients (LON4021) was enrolled into the lower airway subgroup and underwent LAPD measurements – these results are discussed in Chapter 6 (section 5.7.3.1). The demographics, *CFTR* genotype and individual NPD parameter measurements of the patients excluded from the nasal subgroup are displayed in Table 4.5.

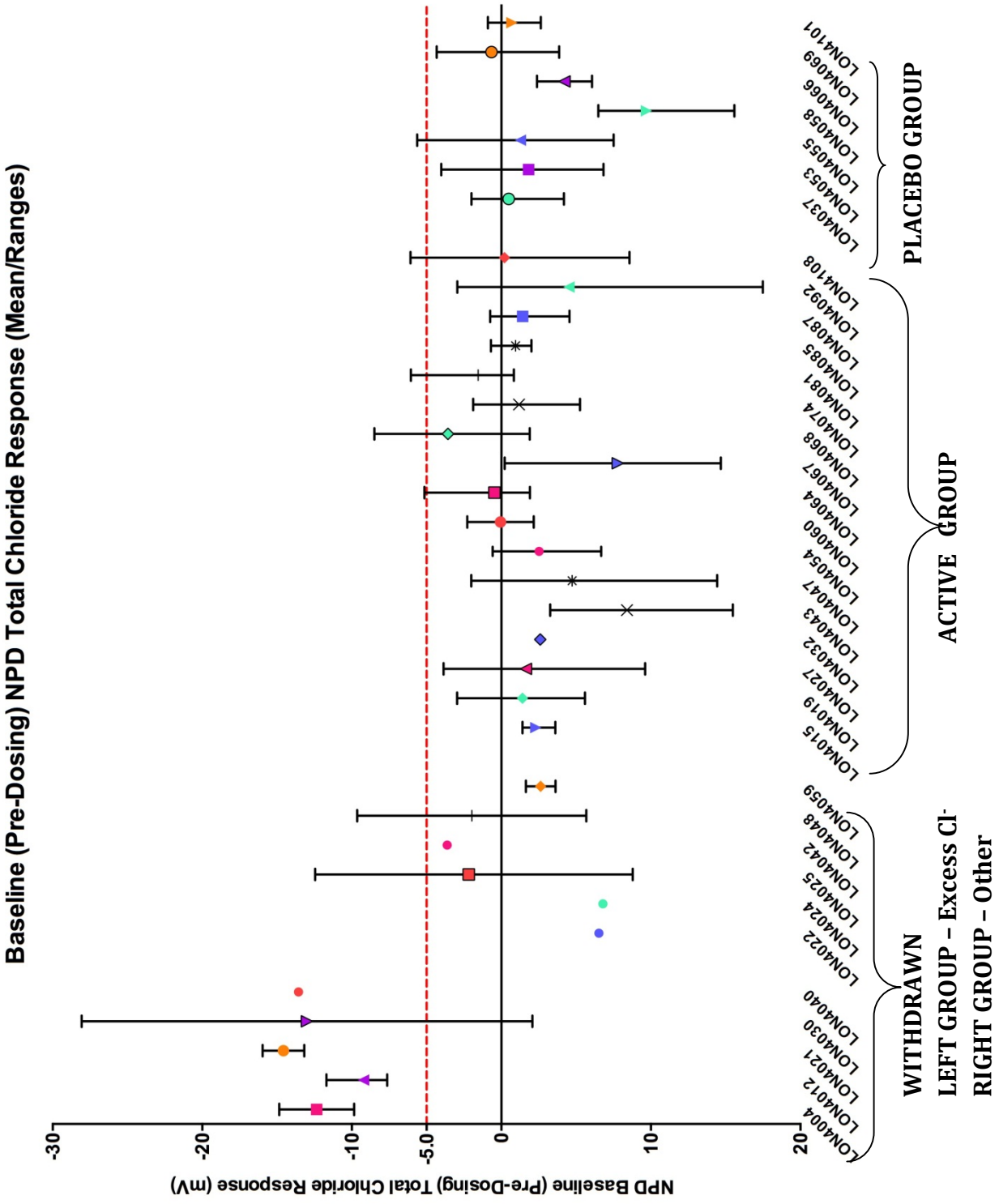


Figure 4.8. Graph displaying mean (range) total NPD chloride responses for all patients enrolled into the nasal subgroup; patients have been grouped into pre-dosing withdrawals and treatment arm (intention to treat population). Red, dashed line delineates the cut-off for exclusion from nasal subgroup.

Trial ID: LON-	Sex/ Age (yr)	Age at Diagnosis	ppFEV$_1$ (%)	Pancreatic Function	*CFTR* Mutation				Mean Potential Difference (mV)				
					Arm 1	Class	Arm 2	Class	Basal	dAmil	dZCA	dZCAI	tCl$^-$
4004	M 31	5	60.9	PI	F508del	2	S549N	3	-80.0	51.8	-8.7	-3.7	-12.4
4012	M 64	39	73.6	PI*	F508del	2	L165S	UNK (Missense)	-44.0	29.3	-7.5	-1.8	-9.2
4021	F 30	16	82.8	PS	F508del	2	3849+10kbC->T	5	-52.5	50.7	-8.5	-6.1	-14.6
4030	M 47	30	67.0	PI	F508del	2	F508del	2	-68.8	41.5	-11.2	-1.8	-13.0
4040	M 63	50	57.1	PI	F508del	2	2789+5G->A	5 (Splice)	-81.8	61.2	-0.4	-13.2	-13.6

Table 4.5. Demographics, CFTR genotype and nasal potential difference measurements for 5 patients excluded from nasal subgroup owing to excessive (>5 mV) total chloride secretion. ppFEV1 – percent predicted FEV$_1$ (at screening); tCl$^-$ - total chloride response. Patient developed pancreatic exocrine failure aged 63 yr.

4.3 NPD Response to Repeated Non-Viral Gene Therapy

The phases of each NPD were analysed, looking for changes in (i) sodium transport (correction of ENac regulation was considered unlikely based on the published literature relating to the degree of cellular correction required to achieve this), and (ii) chloride transport (direct assessment of CFTR function).

The data presented are for the per protocol patients only (i.e. receiving ≥9 doses), unless specifically mentioned. Baseline values are presented as the mean of up to 3 pre-dosing measurements, with follow-up values being presented as the mean of 2 post-dosing measurements. The results focus on the change from pre to post-doing, looking at changes in PD following 12 months treatment with gene therapy. NPD measurements made at intervals throughout the trial (14 or 28 days following dose 3, 6 and 9) have not been specifically included in this results section, other than when individual patients are discussed. The author has chosen specifically not to analyse or discuss these results, but during the overall NPD analysis the interval results did not significantly differ from the overall follow-up data, nor provide additional information on the efficacy of gene therapy.

4.3.1 Assessment of sodium transport by NPD in response to gene therapy

4.3.1.1 Basal Nasal Potential Difference

Pre-treatment, the basal PD in the active treatment and placebo group was similar (p=0.67) and in the range expected for patients with CF. If demonstrating an improvement, the basal measurement would depolarise, i.e. change by a positive value. No significant change in basal PD was seen in the active treatment group in response to 12 nasal doses of gene therapy when compared to both pre-dosing values (p=0.16, paired t-test); nor was there a statistical difference at the

end of the trial for the basal PD between the active and placebo group at follow-up (p=0.79), with both groups recording similar values.

The pre- and post- dosing values, and the change in basal PD are tabulated and shown graphically in Table 4.6 and Figure 4.9. From observation of the graphs alone it is, however, obvious that some individual subjects have noticeable changes in their basal PD at follow-up (both treatment groups) – these will be further considered in section 4.3.1.3.

	Placebo Group (n = 6)	**Active Treatment (n = 15)**
Baseline basal (mV)		
Mean (SD)	-51.4 (18.3)	-54.7 (13.6)
Follow-up basal (mV)		
Mean (SD)	-45.8 (18.5)	-47.0 (14.9)
Change in basal from baseline (mV)		
Mean (SD)	5.5 (13.6)	7.7 (20.3)
p-value (paired t-test)	0.37	0.16

Table 4.6. Basal NPD at baseline and in response to treatment (active and placebo treatment groups).

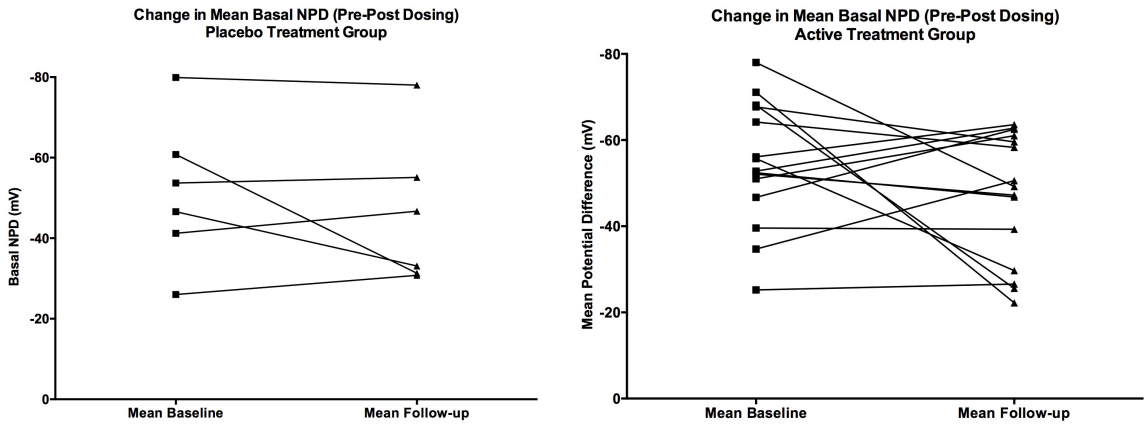


Figure 4.9. Change in individual patient mean basal NPD measurements at baseline and in response to treatment (active and placebo treatment groups).

4.3.1.2 Amiloride response (dAmil)

The mean (SD) response to amiloride (dAmil) at baseline in the active and placebo groups was comparable (p=0.82, paired t-test). A favourable response to gene therapy would be reflected by a reduction in the response to amiloride, again suggesting reduced Na^+ hyperabsorption. There was no significant change in amiloride response (dAmil) in the active treatment group following 12 doses of gene therapy (p=0.13, paired t-test), and no statistical difference between the follow-up dAmil response between the active and placebo groups (p=0.53, paired t-test).

The group data are summarised in Table 4.7, with individual patient data being shown graphically on Figure 4.10. As with the basal PD measurements, individual patients are again noted to have had a large change in amiloride response – these are discussed in section 4.3.1.3.

	Placebo Group (n = 6)	**Active Treatment (n = 15)**
Baseline dAmil (mV)		
Mean (SD)	31.0 (19.3)	31.2 (11.2)
Follow-up dAmil (mV)		
Mean (SD)	29.5 (17.0)	25.0 (12.1)
Change in dAmil from Baseline (mV)		
Mean (SD)	-1.5 (11.9)	-6.2 (14.8)
p-value (t-test)	0.77	0.13

Table 4.7. Response to amiloride (dAMIL) NPD measurements at baseline and in response to treatment (active and placebo treatment groups).

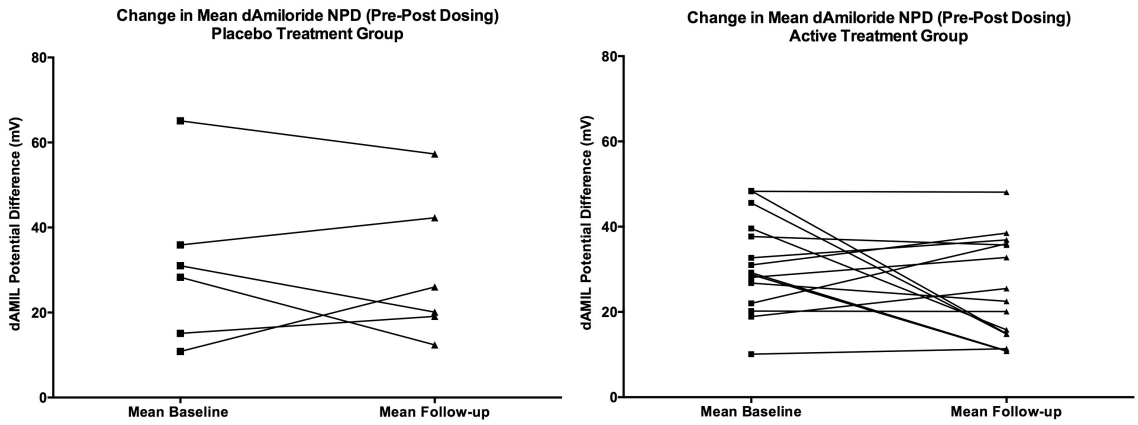


Figure 4.10. Change in individual patient mean dAMIL NPD measurements at baseline and in response to treatment (active and placebo treatment groups).

4.3.1.3 Individual subjects demonstrating a sizable change in basal and dAMIL NPD responses

There is no definition for a 'response' in the two Na^+-related domains of the NPD, and whilst it is proposed that a basal PD of -30 mV may delineate CF from non-CF (typical basal PD values of -50 mV and – 20 mV respectively), there is not an agreed non-CF threshold unlike that for chloride response [204]. Whilst as a group, no significant difference in Na^+ transport is observed in response to active or placebo treatment, individual patients were noted to have a sizeable change (Figures 4.9 and 4.10), with 3/15 (20%) patients demonstrating a change in basal PD into a 'non-CF range' following active treatment.

The author has chosen to review patients having had changes in basal and dAMIL according to the following criteria: he has defined an improvement as a change from mean baseline to mean follow-up of (i) basal >20%; (ii) dAMIL >30%. These magnitudes of change were chosen as they lay outside the baseline mean *CV* of NPD measurements and therefore any change greater than this would be less likely to be a result of the natural variability of the test.

This approach has identified 7 patients (from both treatment groups) with a marked changed in basal PD or dAMIL, and includes patients where the nasal PD has moved from a CF into a non-CF 'range'. Six of these patients had marked

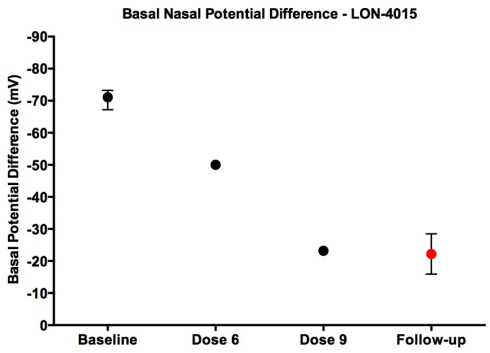
changes in both basal PD and dAMIL, which would not be unexpected given that they are both indices of Na^+ transport. Analysing only this 'responder' population, the change from pre- to post-treatment in the active treatment group is significant for basal PD (p=0.02) and dAMIL (p=0.02).

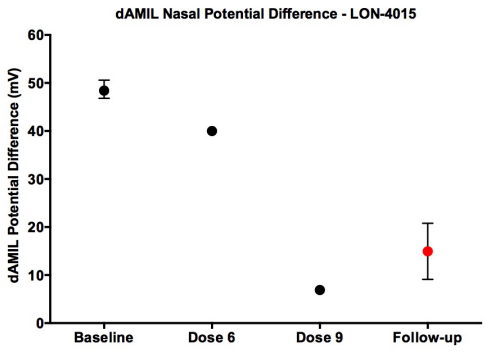
The data are summarised in Table 4.8, which includes relevant comments on the clinical status of the patient at the time of follow-up, as a change in the nasal mucosa may attenuate the ion transport.

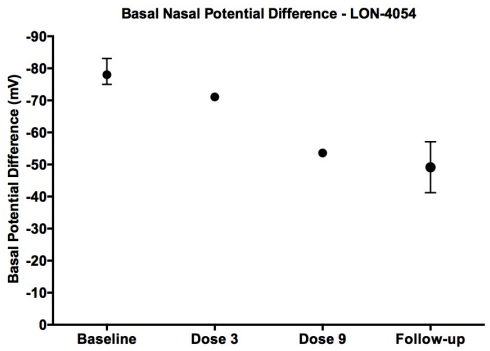
To understand whether the changes observed in basal PD and dAMIL from baseline to follow-up were seen throughout the entire study period, each patient's basal and dAmil PD measurements taken during the study have been plotted and are shown in Figure 4.11 (active subjects Figures 4.11 *a-e*; placebo group Figures 4.11 *f & g*).

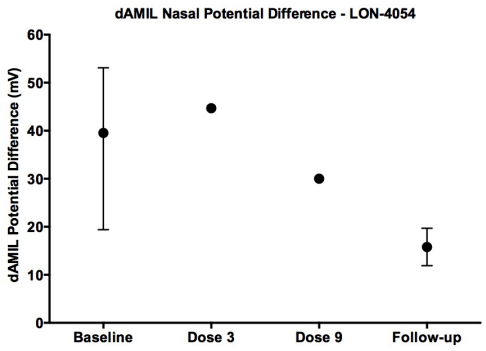
Table 4.8. Table summarising MDT Subjects displaying a change in basal NPD at follow-up of >20% of baseline (active and placebo treatment groups).

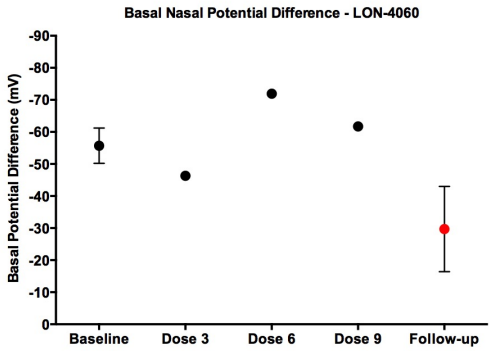
Subject ID		LON4015	LON4054	LON4060	LON4068	LON4081	LON4055	LON4069
	Treatment Group	Active	Active	Active	Active	Active	Placebo	Placebo
Mean Basal NPD (mV)	Baseline	-71.1	-78.0	-55.7	-52.1	-68.1	-60.8	-46.6
	Follow-up	-22.2	-49.2	-29.7	-47.2	-25.6	-31.3	-33.1
	Percentage change (%)	68.8	36.9	46.7	9.4	62.4	48.5	29.0
Mean dAMIL (mV)	Baseline	48.4	39.6	28.2	29.2	17.8	28.3	31.0
	Follow-up	14.9	15.8	10.8	10.9	14.8	12.4	20.1
	Percentage change (%)	69.2	60.1	61.7	62.7	16.9	56.2	35.2
Comments		Hayfever at Follow-up visits	Nil	Nasal congestion at 1st follow-up visit	Sinusitis at follow-up	URTI at FU-1; FU-2 performed 9-weeks following dose 12	Nasal congestion at FU-2	Nil











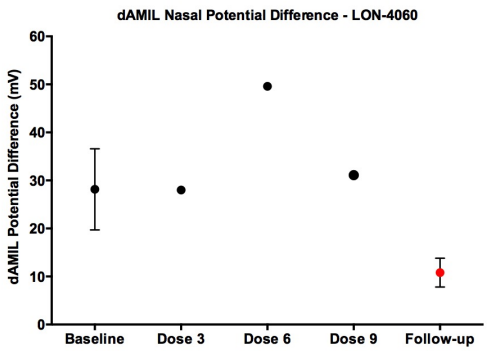


Figure 4.11 (a-c). Paired graphs displaying change in basal (mean/range) PD and change in dAMIL (mean/range) from baseline in ACTIVE treated subjects (LON-4015, LON-4054 & LON-4060). Red data points denote intercurrent acute nasal symptoms at time of measurement.

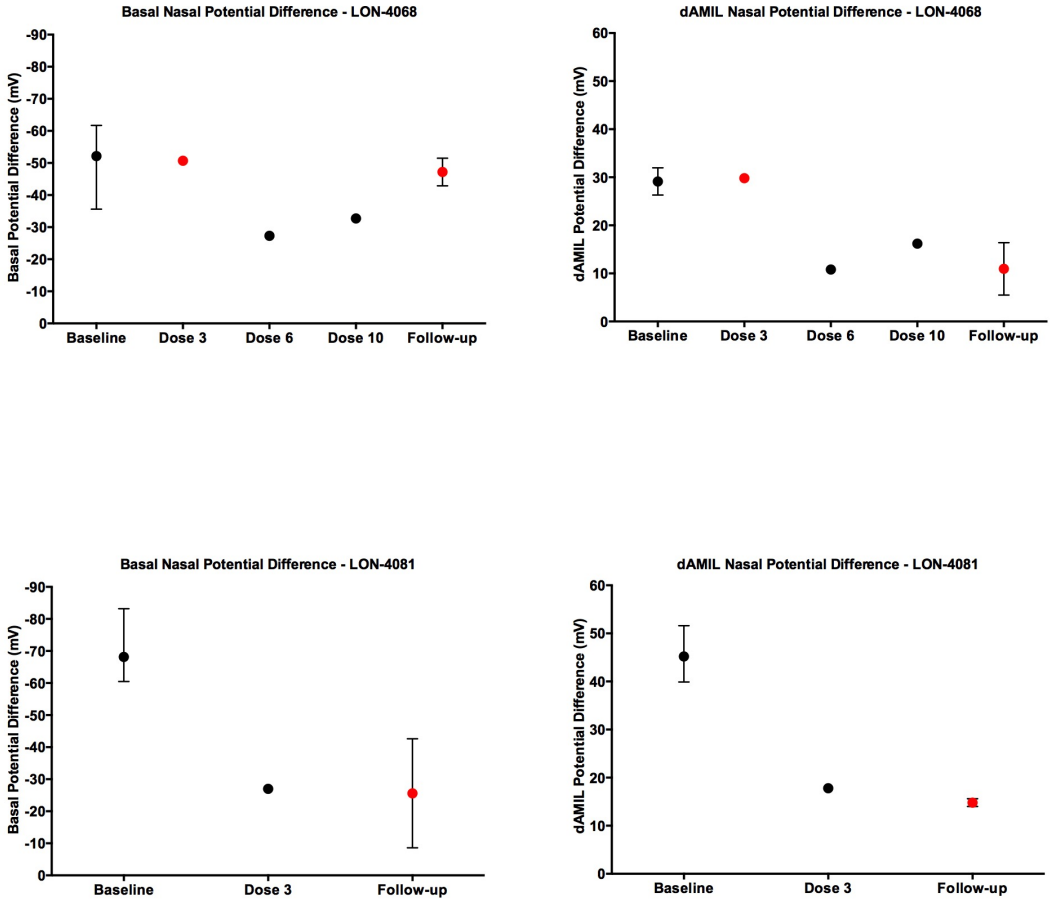


Figure 4.11 (d,e). Paired graphs displaying change in basal (mean/range) PD and change in dAMIL (mean/range) from baseline in ACTIVE treated subjects (LON-4068 & LON-4081). Red data points denote intercurrent acute nasal symptoms at time of measurement.

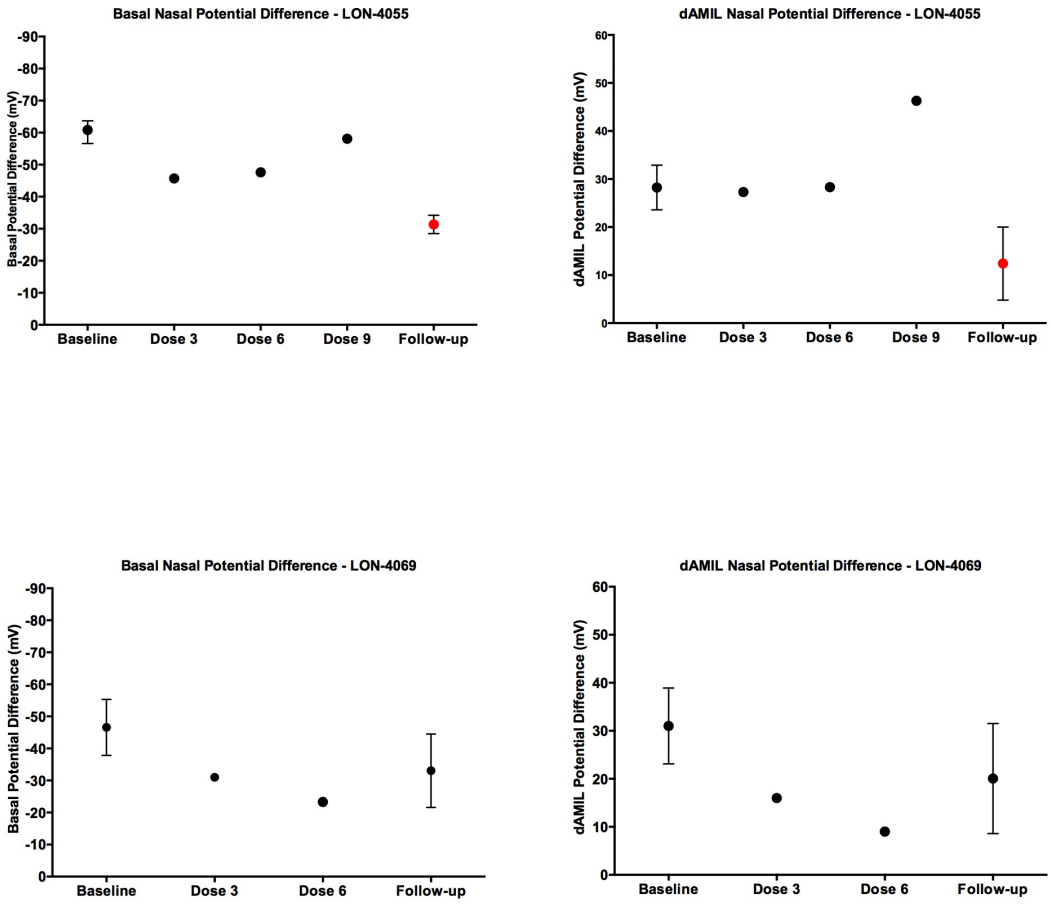


Figure 4.11 (f,g). Paired graphs displaying change in basal (mean/range) PD and change in dAMIL (mean/range) from baseline in PLACEBO treated subjects (LON-4055 & LON-4069). Red data points denote intercurrent acute nasal symptoms at time of measurement.

4.3.2 Assessment of chloride transport by NPD in response to gene therapy

Successful gene transfer that results in a functioning CFTR protein should lead to detectable Cl^- transport. At baseline, all CF patients should exhibit almost no change in PD in response to stimulation of Cl- secretion, reflecting the absence of Cl^- movement; amelioration of CFTR function would thus be demonstrated by a negative change in PD at this phase of the measurement.

At baseline, the passive, stimulated and total chloride responses between the active and placebo groups were not significantly different. Results pertaining to Cl^- transport in response to treatment have been separated into passive transport and that stimulated by isoprenaline, and the combined (total) Cl^- response which will then be used for all future comparisons and discussions within this thesis. There was no pre-defined threshold to delineate a treatment 'response' with regards to chloride transport, but PD changes in the non-CF direction were considered meaningful.

4.3.2.1 Chloride transport in response to ZCA solution

No significant change was seen in mean NPD response to a ZCA solution following 12 doses of gene therapy, with no significant difference in mean dZCA response from baseline to follow-up in the actively treated group (p=0.85, Wilcoxon rank test) nor between follow-up measurements between the active and placebo groups (p=0.75, Wilcoxon rank test). The group summaries are shown in Table 4.9, with the change in individual dZC responses in response to treatment in each group being displayed graphically in Figure 4.12.

	Placebo Group (n = 6)	**Active Treatment (n = 15)**
Baseline (Pre-Dosing) dZCA (mV)		
Median (IQR)	-0.1 (-0.9, 3.6)	1.5 (0.0, 3.0)
Follow-up (Post-Dosing) dZCA (mV)		
Median (IQR)	0.6 (-0.7, 2.5)	1.6 (-0.7, 2.6)
Change in dZCA from Baseline (mV)		
Median (IQR)	0.4 (-1.6, 1.6)	0.2 (-2.4, 1.2)
p-value (Wilcoxon)	0.84	0.85

Table 4.9. Response in mean dZCA NPD measurements at baseline and in response to treatment (placebo and active treatment groups).

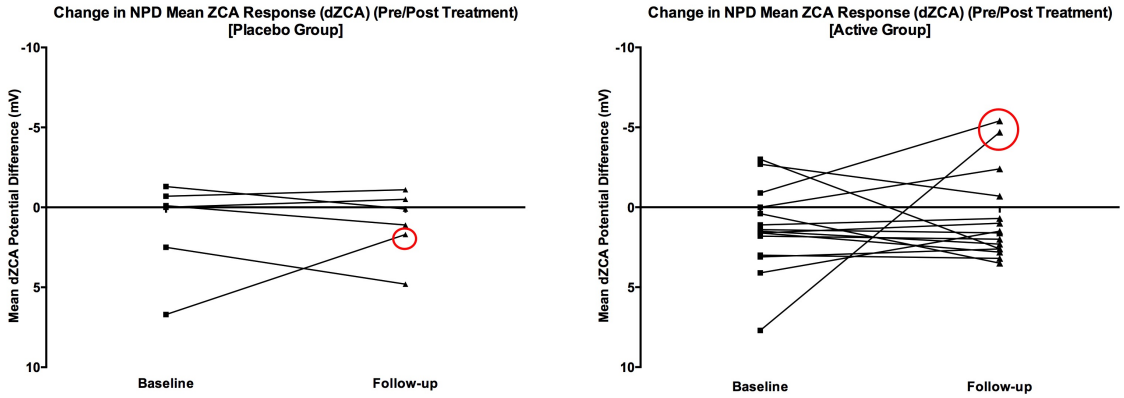


Figure 4.12 - Paired graphs demonstrating change in mean dZCA response from the placebo (left) and active (right) treatment groups. Subjects circles in red denote a sizable dZCA response.

When studying the change in response to ZCA for individual subjects from baseline to follow-up (Figure 4.12), it is apparent that 3 subjects (2 active group, 1 placebo) have a larger increase in the mean dZCA than the rest of the group (placebo subject: LON-4058 -5.0 mV; active subjects: LON4064 -4.4 mV, LON4067 -12.3 mV). These individual patients had improved dZCA values throughout the trial, except at time-points corresponding to an intercurrent URTI (data points in red), shown in Figure 4.13.

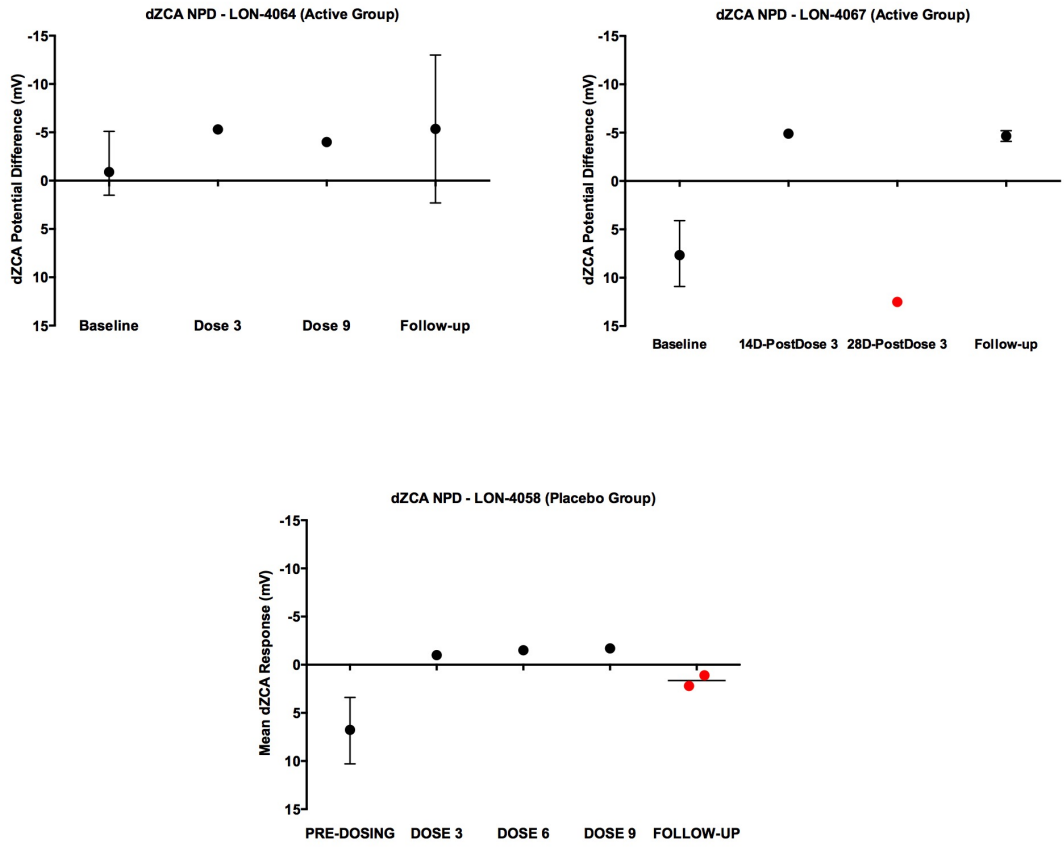


Figure 4.13. Mean (range) dZCA response of patients throughout the MDT (i) TOP: active – LON4064 and LON4067, and (ii) BOTTOM: placebo – LON-4058. The time point refers to the last dose received, with the NPD being performed ≈28-days later. The red data points denote intercurrent acute nasal symptoms (congestion) at time of measurement.

4.3.2.2 Stimulated chloride response (in response to ZCAI solution)

No significant change was seen in NPD response to a ZCAI solution following 12 doses of gene therapy (p=0.34, t-test), nor was there a statistical difference in response to treatment between the treatment groups (p=0.15). Small changes from baseline are seen which are comparable to those seen in the placebo group; the range of follow-up dZCAI PD responses was also similar in the two groups. This is summarised in Table 4.10 and in Figure 4.14.

	Placebo Group (n = 6)	Active Treatment (n = 15)
Baseline (Pre-Dosing) dZCAI (mV)		
Mean (SD)	1.4 (1.0)	0.1 (0.9)
Follow-up (Post-Dosing) dZCAI (mV)		
Mean (SD)	0.0 (1.8)	1.0 (3.2)
Change in dZCAI from Baseline (mV)		
Mean (SD)	-1.4 (1.7)	0.9 (3.5)
p-value (t-test)	0.11	0.34

Table 4.10. Response in mean dZCAI NPD measurements at baseline and in response to treatment (placebo and active treatment groups).

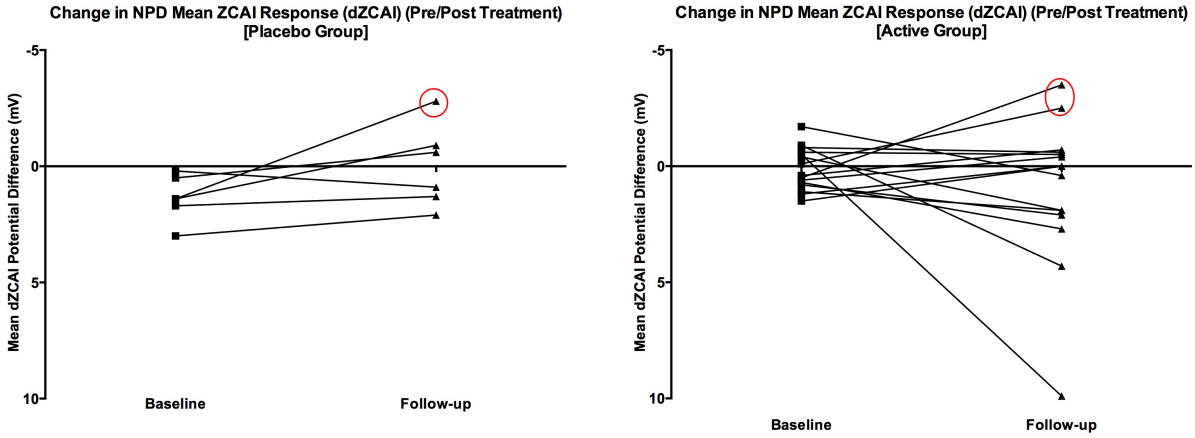


Figure 4.14. Paired graphs demonstrating change in mean dZCAI response from the placebo (left) and active (right) treatment groups. Subjects circled red highlight a sizable response compared to others.

Whilst the magnitude of response to ZCAI is less than that seen in the response to ZCA, 3 different subjects are noted to have a sizeable response greater than others (2 active, 1 placebo groups): the mean dZCAI change from baseline to follow-up is: active: LON-4027 -4.0 mV and LON-4087 -2.4 mV; placebo: LON-4101 -4.2 mV. Each of these patient's ZCAI responses, measured throughout the MDT, were analysed and significant variation was seen (Figure 4.15).

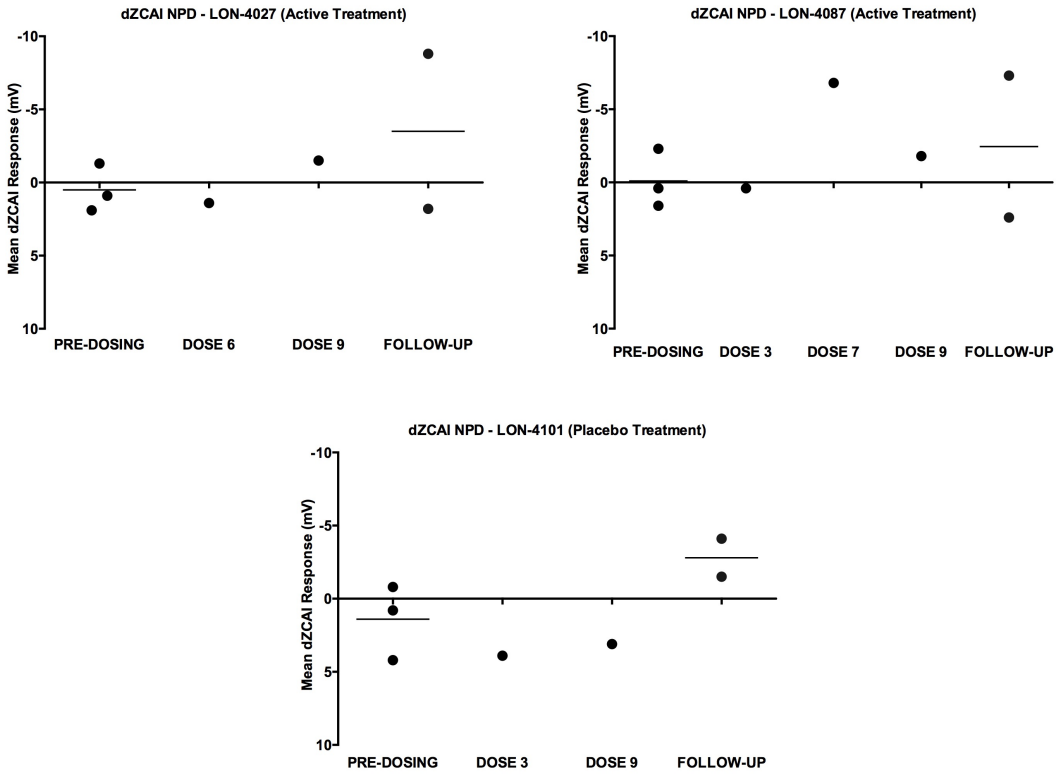


Figure 4.15. Mean (range) dZCAI response of patients throughout the MDT (i) TOP: active – LON4027 and LON4087, and (ii) BOTTOM: placebo – LON4101. The time point refers to the last dose received, with the NPD being performed ≈28-days later.

4.3.2.3 Total chloride response (tCl^-)

The tCl^-, a sum of the dZCA and dZCAI, is a calculated change in PD and reflects the total Cl^- efflux in 10 min; hyperpolarisation (i.e. values becoming more negative), would support CFTR-mediated chloride transport.

There was no statistical difference between baseline (pre-treatment) tCl^- responses between the two groups. No treatment effect was detected in response to 12 doses of nasal gene therapy, at either 14-days or 28-days post-dose 12 (data not shown), or from the mean of both follow-up visits. Whilst improvements in mean tCl^- were noticed in the treatment group (paired analysis), with several NPD measurements settling in the non-CF range, similar changes were seen in the placebo group and no significant treatment effect was detected between the two groups (p=0.58). The largest individual tCl^- response

was demonstrated by subject LON-4064 (30M, *Phe508del/Phe508del*) in the active treatment group, with a measured response of -10.3 mV (14-days post-dose 12).

As there was no difference between results at 14- and 28-days post-dose, the combined follow-up results are used for the remainder of this thesis. These results are shown for (i) the group (Table 4.11) and (ii) for individual subjects (Figure 4.16).

	Placebo Group (n = 6)	**Active Treatment (n = 15)**
Baseline (Pre-Dosing) tCl^- (mV)		
Mean (SD)	2.4 (3.8)	1.6 (2.8)
Follow-up (Post-Dosing) tCl^-(mV)		
Mean (SD)	1.0 (3.6)	1.7 (4.2)
Change in tCl^- from Baseline (mV)		
Mean (SD)	-1.4 (3.6)	0.1 (5.9)
p-value (t-test)	0.39	0.95

Table 4.11. Response in mean tCl- NPD measurements at baseline and in response to treatment (placebo and active treatment groups).

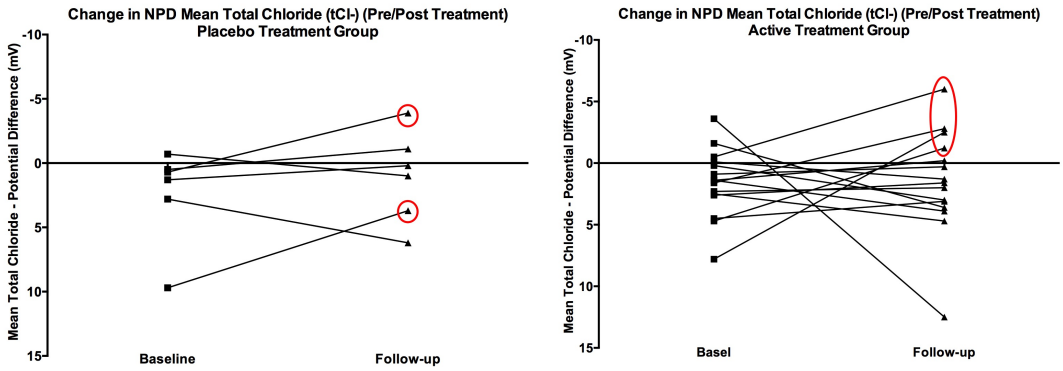


Figure 4.16. Paired graphs demonstrating change in mean total Cl^- response (tCl^-) dZCAI response from the placebo (left) and active (right) treatment groups. Subjects demonstrating noticeable improvement in tCl^- response are circled red.

Six subjects (4 (26.7%) active treatment; 2 (33.3%) placebo group) demonstrated a noticeable improvement in tCl^- (highlighted by red circles in Figure 4.16) compared with baseline: 4 of the 5 patients demonstrating

improvement towards non-CF values (i.e. 0 to -5 mV) were treated by gene therapy, with one of these patients entering into the non-CF range (patient LON-4064, tCl^- = -6.0). The tCl^- responses throughout the trial for these subjects are graphically plotted and are shown in Figure 4.17 (active group graphs *a-d*; placebo group graphs *e-g*); subject LON4055 (placebo group) has also been included as whilst not demonstrating improvement at follow-up, the on-treatment values appeared to demonstrate increased tCl^- transport.

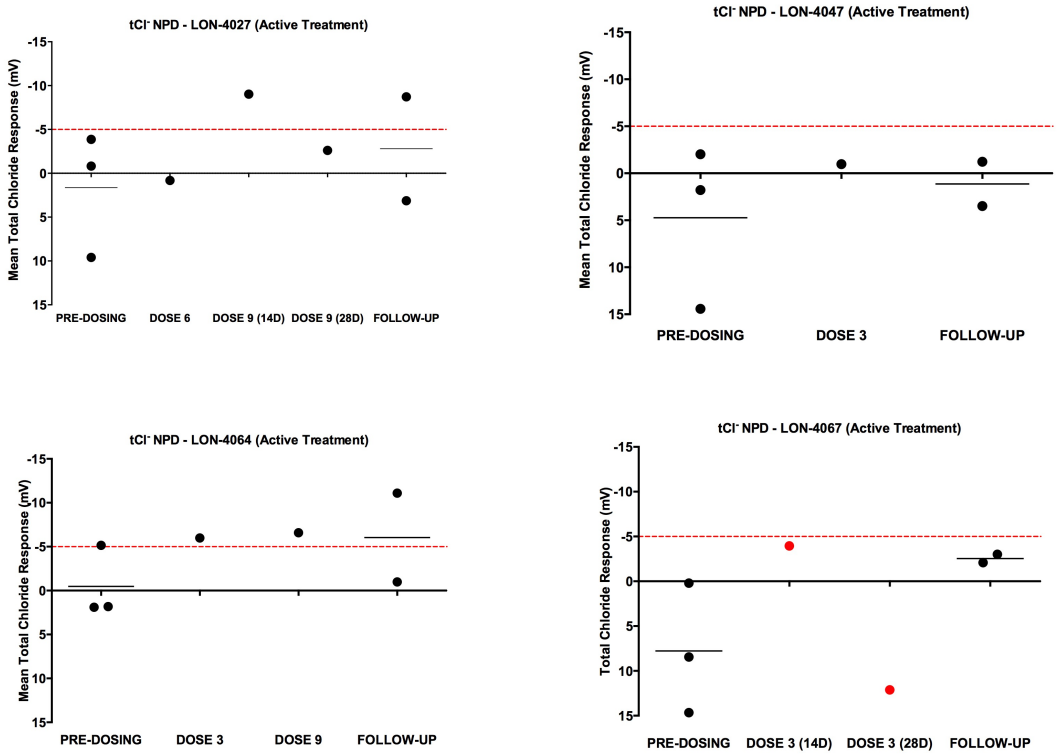


Figure 4.17 (*a-d*). Mean (range) of tCl^- response of 'responders' (ACTIVE group). The time point refers to the last dose received, with the NPD being performed ≈28-days later. The transverse red line denotes the -5 mV CF/non-CF diagnostic threshold. Red points denote intercurrent acute nasal symptoms at time of measurement.

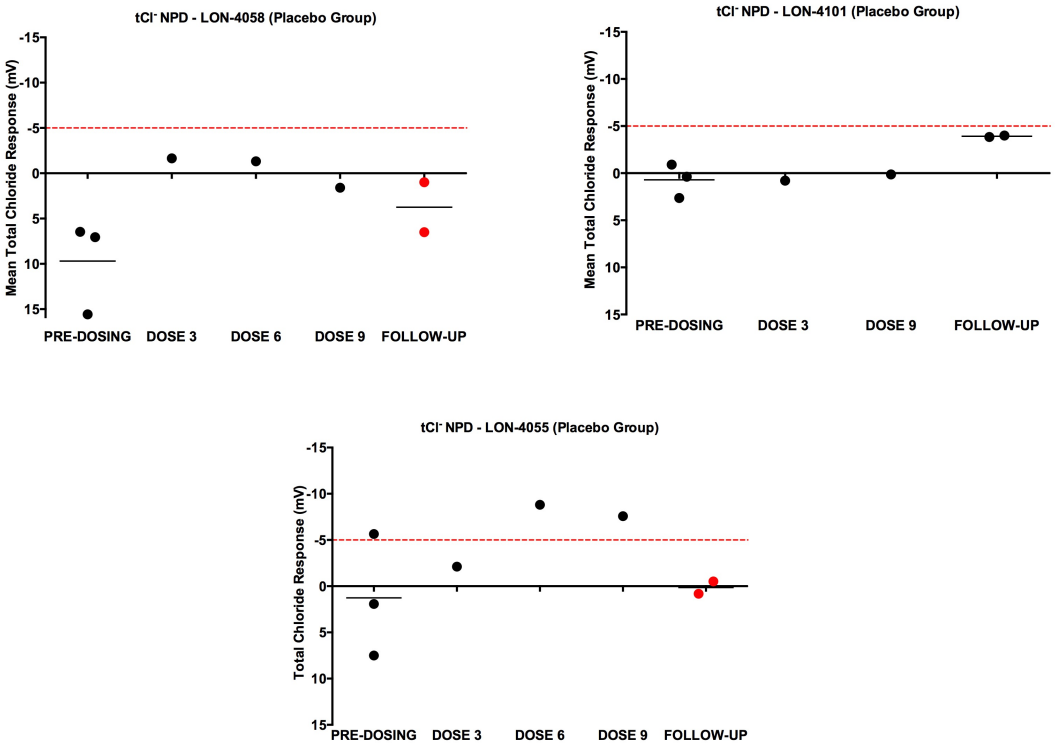


Figure 4.17 (*e-g*) - Mean (range) of tCl^- response of 'responders' (PLACEBO group). The time point refers to the last dose received, with the NPD being performed ≈28-days later. The transverse red line denotes the -5 mV CF/non-CF diagnostic threshold. Red points denote intercurrent acute nasal symptoms on at least one time of measurement.

4.3.2.4 Adjudication of NPD total chloride responses

A further triage of the quality of the traces from both treatment groups was undertaken prior to submission for scientific publication of the trial manuscript. The method used differed slightly from the previous computerised analyses, adopting a traditional approach to PD interpretation. Individual NPD traces were printed from Excel and remained blinded to pre/post measurement and treatment arm. Traces were removed from the analysis if the quality of the trace was too unstable or not interpretable owing to electrical artefact or interference. By this method, 5 patients (4 active, 1 placebo) did not have matched pre/post-dosing values and were removed from the final published analysis.

The author of this thesis unblinded and collated the traces, to allow for data analysis; the mean of every subject's tCl^- responses was calculated, and the response to treatment assessed. Neither method of analysis demonstrated a treatment effect in response to gene therapy: 'first PD analysis' by original computer method = +1.9 mV (p=0.58); 'second PD analysis' by global overview of variability in tracing method = +1.3 mV (p=0.26). The results of both analyses are summarised in Table 4.12 and shown graphically in Figure 4.18.

	'First Analysis' Computer PD Analysis		**'Second Analysis' Visual PD Interpretation**	
	Placebo (n=6)	**Active (n=15)**	**Placebo (n=5)**	**Active (n=11)**
Mean (SD), mV	-1.4 (3.6)	-0.1 (6.0)	-1.2 (3.7)	0.7 (1.7)
Treatment Effect, mV	1.3 (p=0.58)		1.9 (p=0.26)	

Table 4.12. Table summarising mean (SD) NPD tCl^- response in response to placebo and active treatment, and treatment effect, by two methods of analysis: (i) first analysis by computer, (ii) second analysis by visual trace interpretation. Results of unpaired t-test shown.

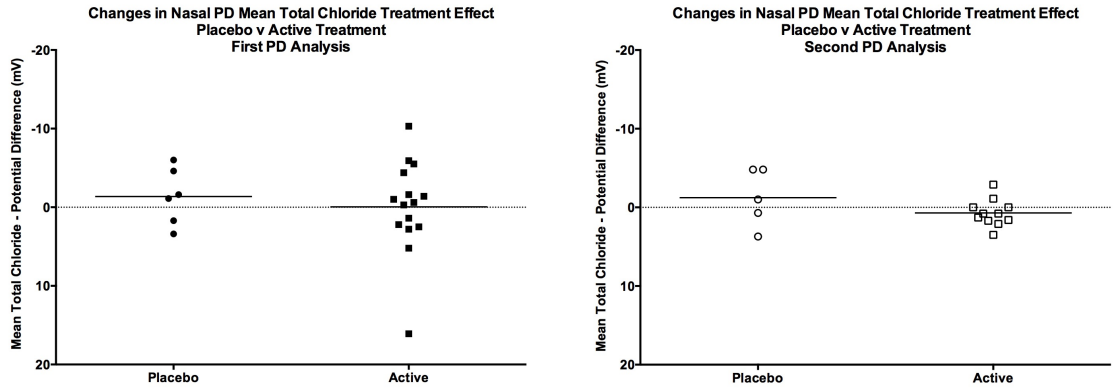


Figure 4.18. Graphs summarising the change in individual subject's mean tCl^- (NPD) in response to placebo and active treatment, and the mean group treatment response as analysed by (i) LEFT – first analysis by computer, n=6 (placebo), n=15 (active); and (ii) RIGHT – second analysis by visual trace interpretation, n=5 (placebo); n=11 (active).

4.3.3 Further assessments of chloride responses

The criteria outlined in section 4.1.3.2 were applied to the results from the MDT using follow-up data from 14 and 28 days post-final treatment dose as an investigative method to assess chloride responses. Each parameter used on the follow-up NPD data was calculated by the author (computer method of analysis) for both treatment groups to determine whether any method could discriminate a treatment effect over placebo – a summary of these analyses is shown in Table 4.13.

This summary demonstrates that no method applied in isolation is able to identify an electrophysiological treatment response to gene therapy as defined by the degree of chloride movement. As no gold standard exists it is difficult to compare apparent responses against a standard.

Neither NPD equations could detect a treatment response. At baseline, all pre-dosing values were confirmed to be 'non-CF' using both equations. At follow-up only 1 NPD had demonstrated a transformation from CF to non-CF: LON4027 at FU-1, with a tCl^- response of -8.8 mV and a modestly small dAMIL of 20.3 mV: this patient had a similar shift into a non-CF range 14 days following dose 9, with only 1 patient having a similar response through the duration of the trial (LON4081 – 28 days post-dose 3 with gene therapy).

			GREATER ANY PRE-DOSE TEST?	CHANGE FROM MEAN PRE-DOSE TO MEAN POST-DOSE >5mV*	ABSOLUTE CL SECRETION >5mV	WILSCHANSKI EQUATION	SERMET EQUATION
Active Group (n=15)	Follow-up 1 n, %	ZCA	4 (26.7)	-	0 (0.0)	0 (0.0)	0 (0.0)
		ZCAI	4 (26.7)	-	1 (6.7)	0 (0.0)	0 (0.0)
		Total Cl-	3 (20.0)	-	1 (6.7)	1 (6.7)	0 (0.0)
	Follow-up 2 (or total mean) n, %	ZCA	5 (33.3)	2 (13.3)	2 (13.3)	0 (0.0)	0 (0.0)
		ZCAI	2 (13.3)	0 (0.0)	1 (6.7)	0 (0.0)	0 (0.0)
		Total Cl-	3 (20.0)	3 (20.0)	1 (6.7)	0 (0.0)	0 (0.0)
Placebo Group (n=6)	Follow-up 1 n, %	ZCA	1 (16.7)	-	0 (0.0)	0 (0.0)	0 (0.0)
		ZCAI	1 (16.7)	-	0 (0.0)	0 (0.0)	0 (0.0)
		Total Cl-	1 (16.7)	-	0 (0.0)	0 (0.0)	0 (0.0)
	Follow-up 2 (or total mean) n, %	ZCA	1 (16.7)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
		ZCAI	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		Total Cl-	3 (50.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)

Table 4.13. Summary of the number (percentage) total responses of each chloride phases for all follow-up NPD at visits 1 (14 ± 2 days) and 2 (28 ±2 days) for active and placebo groups, compared to pre-dosing (baseline) measurements. Follow-up 1 and 2 are shown separately, except for *when only mean pre-/post-dosing values are analysed.

4.4 Quantification of Transgene DNA and mRNA in the Nasal Epithelium

Nasal brushing samples were taken from both nostrils at pre-dosing and at follow-up visits from 23 patients in the intention to treat nasal subgroup (placebo = 6, active = 17).

Transgene *CFTR* DNA and mRNA were quantified as described in Chapter 3, section 3.4.3. Quantification of *CFTR* was made on positive samples, and a percentage of pGM169-specific *CFTR* to genomic *CFTR* calculated; when positive levels fells below the range for limit of quantification (LOQ) or quantification of endogenous *CFTR* was negative, the result was recorded as positive but not quantifiable (PBNQ). 2 samples from the placebo group failed analysis owing to technical reasons and are recorded as not determined (ND).

Transgene specific DNA was detected in the following proportions of patients: (i) placebo (n=6): pre-treatment, 4 patients had no DNA detected, and 2 samples were reported as ND; post-treatment, 3 (50%) patients had DNA detected in their nasal epithelium and is likely a result of sample contamination within the laboratory, 1 (17%) patient had no DNA identified, and the same two patients had ND results; (ii) active (n=17): pre-treatment, 13 (76%) patients had no DNA detected, 2 (12%) had low levels of DNA measured, and 2 (12%) samples were reported as PBNQ; post treatment, 16 (94%) patients had DNA detected (with increased DNA being measured in the patients positive or PBNQ at baseline), the sample from 1 (6%) patient was reported as PBNQ. A statistical difference was measured between the DNA measured in the active to placebo group between baseline and follow-up (p=0.04, Mann Whitney test). The change in *CFTR* DNA in response to treatment is shown in Figure 4.19.

No vector-specific mRNA was quantifiable in either treatment group at follow-up.

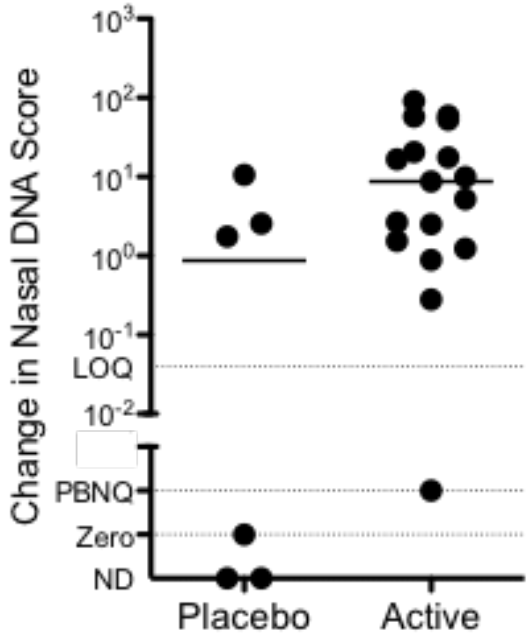


Figure 4.19. Graph summarising changes in the measurements of DNA from nasal brushing from each patient and the median group value (placebo: n=6; active: n=17). LOQ – limit of quantification; PBNQ – positive but not quantifiable (PBNQ); ND – not determined.

4.4.1 Relationship between changes in *CFTR* DNA and NPD chloride efflux

To investigate whether patients with measured increases in *CFTR* DNA had electrophysiological evidence of increased CFTR function, as measured by an increase in NPD total chloride efflux, the two parameters were correlated. The data from the per protocol nasal subgroup (15 patients) were analysed. No correlation was identified between the changes in DNA from the nasal epithelium with the tCl^- measured by NPD in response to active treatment (r=0.14, p=0.61; n=15) (Figure 4.20).

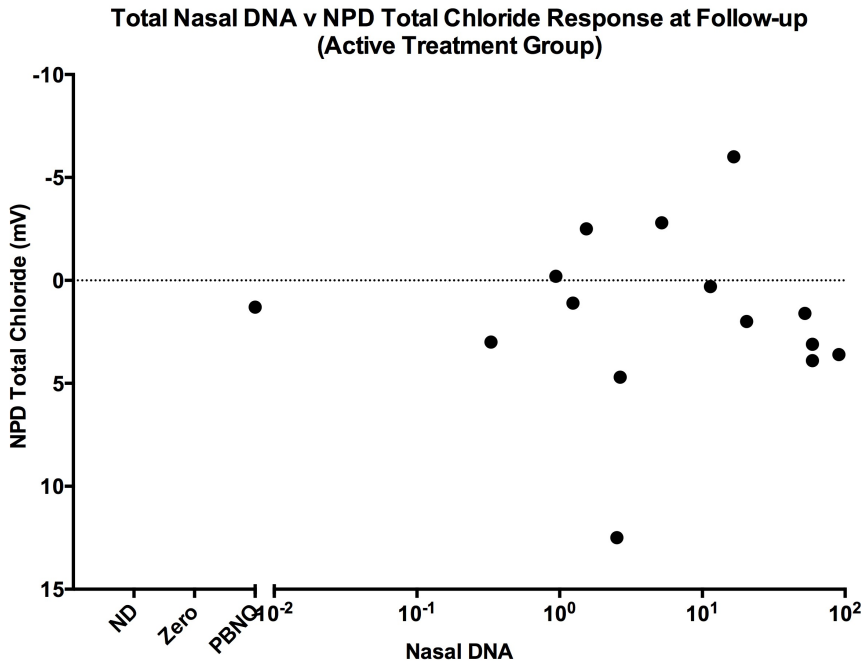


Figure 4.20. Graph plotting the correlation between nasal *CFTR* DNA and NPD measured total chloride at follow-up in response to active treatment (n=15).

4.5 Relationship between nasal airway electrophysiology and measurements of lung function

As NPD is a direct assessment of CFTR function, it is relevant to examine the relationship between changes in airway electrophysiology and measurements of downstream airway function, such as FEV_1 and LCI, to understand whether changes in one parameter demonstrate changes in another.

The following are the results of correlations made between the PD measurements of the nasal epithelium and FEV_1 and LCI at (i) baseline and (ii) in response to treatment.

4.5.1 Relationship between NPD and lung function at baseline

NPD parameters (basal, dAMIL and total chloride response) were correlated with FEV_1 and LCI, all at baseline, and are presented below.

4.5.1.1 Basal NPD measurement with FEV_1 at baseline

A correlation was drawn between the mean basal NPD measurement and mean $ppFEV_1$ measurement at baseline.

At baseline, whilst there was a trend towards a more negative basal NPD with worsening of $ppFEV_1$, this did not reach statistical significance (r=0.22, p=0.31, Pearson correlation) (Figure 4.21).

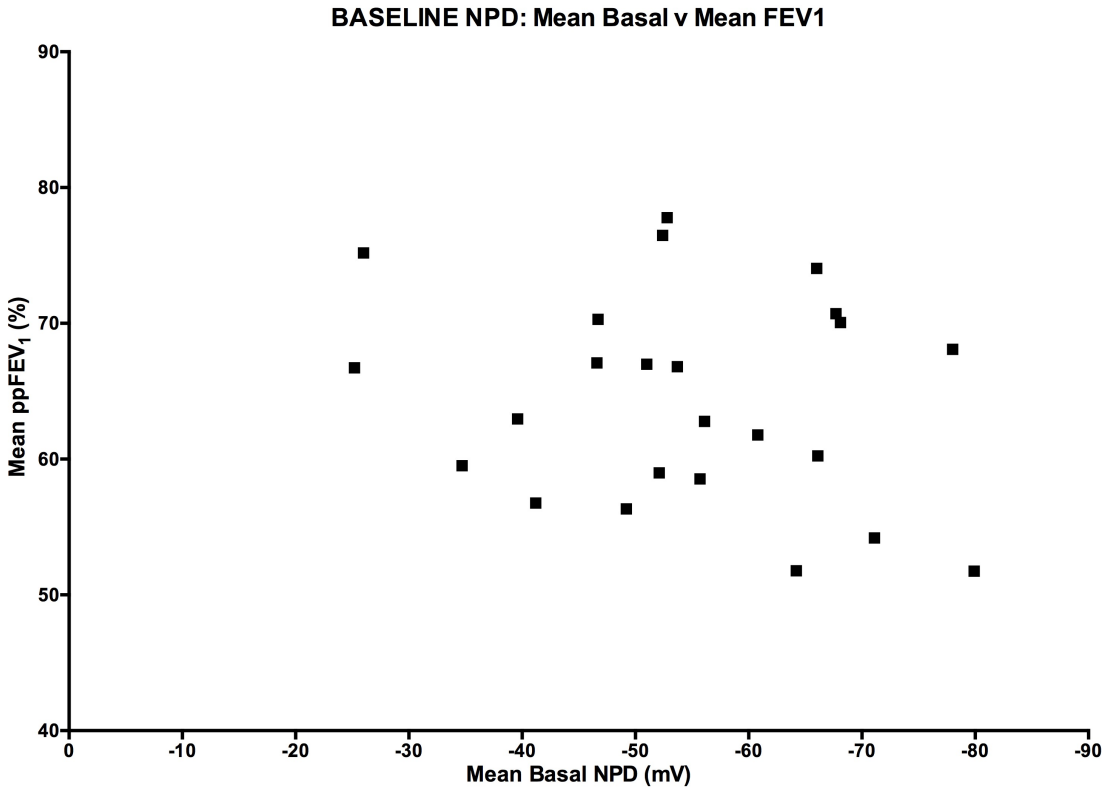


Figure 4.21. Correlation between mean basal nasal PD and mean $ppFEV_1$ at baseline (patients in both groups; n=24).

4.5.1.2 NPD dAmiloride response with FEV_1 at baseline

Mean nasal response to Ringers/Amiloride solution (dAmil) were correlated with mean FEV_1. Both dAMIL and basal PD are parameters of epithelial sodium transport and, as such, a similar relationship to the severity of airway disease

would be expected. At baseline, no correlation was identified between the two parameters (r=-0.37, p=0.08, Pearson correlation) (Figure 4.22).

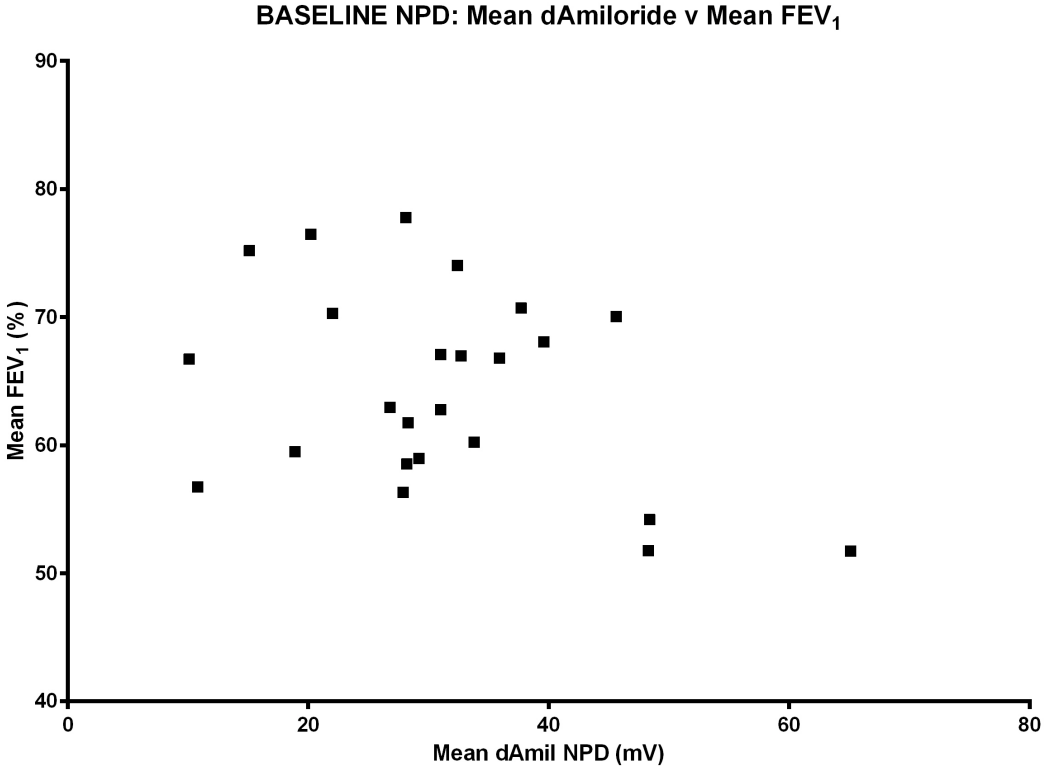


Figure 4.22. Graph showing mean baseline dAmil response against mean baseline ppFEV$_1$ (patients in both groups, n=24).

4.5.1.3 NPD total chloride efflux measurement with FEV$_1$

A weak correlation (r=0.37; p=0.07, Pearson correlation) was observed, although this was in a negative direction, i.e., higher FEV$_1$ being associated with less Cl^- secretion, and therefore counter to expectations. This may be a spurious finding and is further discussed later in this chapter (Figure 4.23).

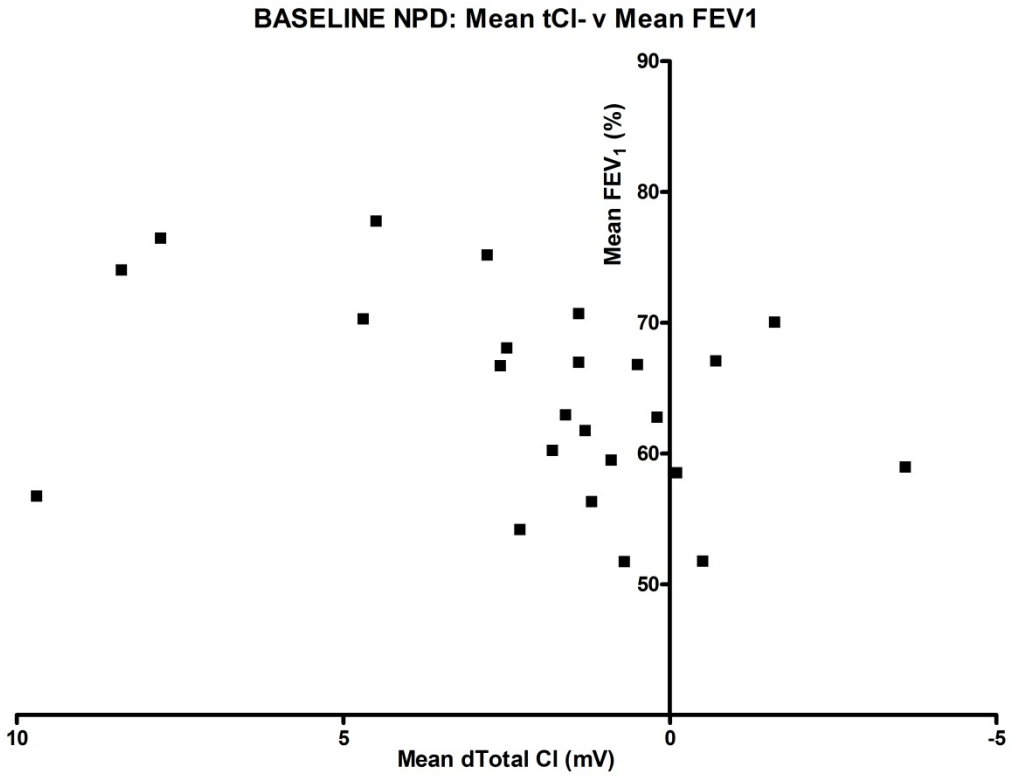


Figure 4.23. Correlation between mean tCl^- nasal PD and mean $ppFEV_1$ at baseline (patients in both groups, n=24).

4.5.1.4 Basal NPD measurement with LCI

Despite a lack of correlation between NPD and FEV_1, LCI is a more sensitive measure of global lung function leading the author to speculate that a relationship might be seen with this parameter. A correlation was thus performed between the mean basal NPD measurements and mean LCI measurements at baseline. One patient (LON-4058), in the placebo arm, had baseline LCI measurements excluded owing to a technical failure during the tracer gas wash-in; therefore the following analyses are of 21 subjects.

At baseline, a moderately strong and statistically significant correlation between mean basal NPD and mean LCI was seen (r=-0.57; p=0.01, Spearman correlation) (Figure 4.24).

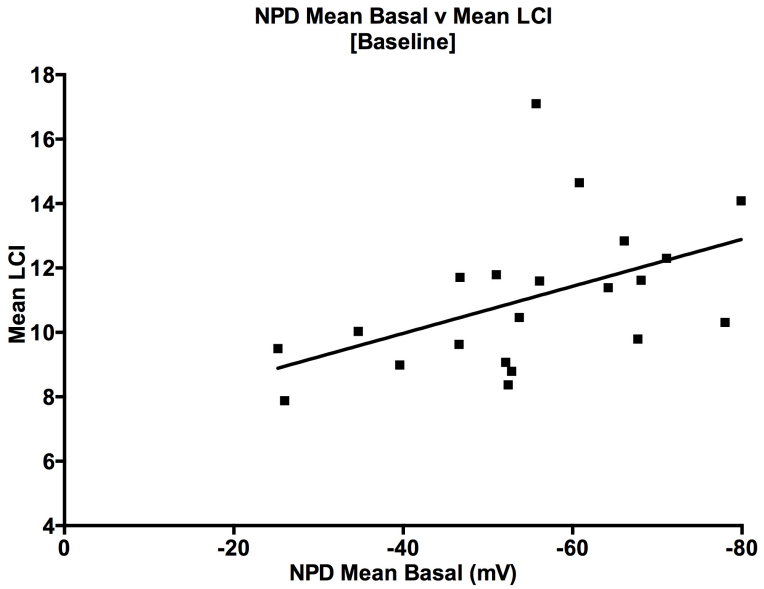


Figure 4.24. Graph demonstrating correlation between mean basal NPD measurement and mean LCI, at baseline (patients in both groups, n=21).

4.5.1.5 NPD dAmilorde response with LCI

A moderate and statistically significant correlation was found between these indices adding support for this previous finding (r=0.51; p=0.02, Spearman correlation) (Figure 4.25).

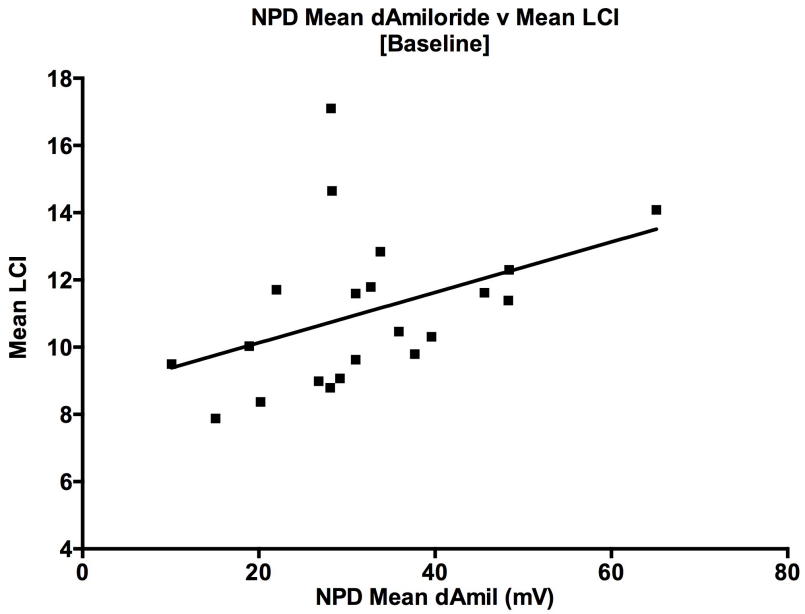


Figure 4.25. Correlation graph between mean baseline dAmil and mean baseline LCI. (Patients in both groups, n=21).

4.5.1.6 NPD total chloride efflux measurement with LCI

In contrast, no correlation was detected between LCI and tCl-, (r=-0.36; p=0.12, n=21) (Figure 4.26).

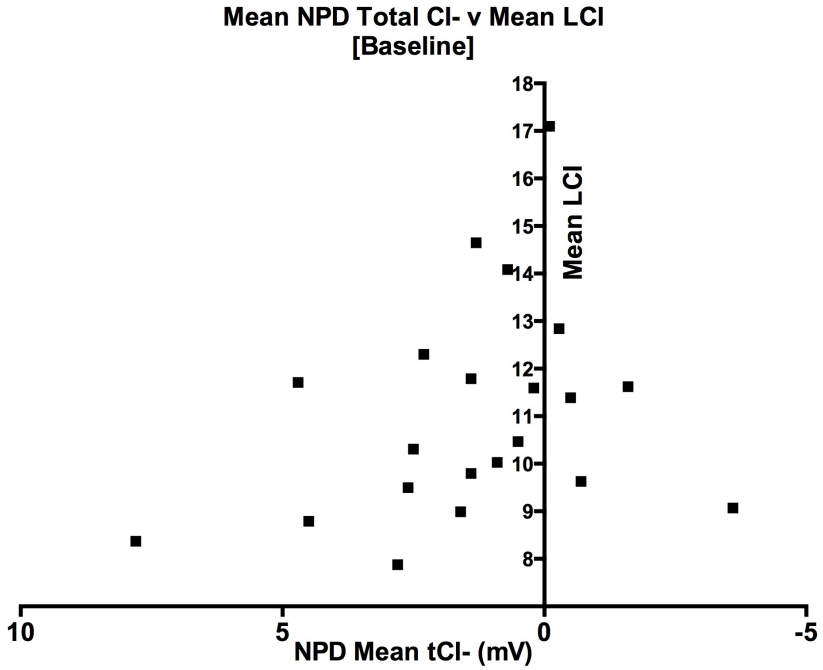


Figure 4.26. Graph showing relationship between mean NPD total chloride (tCl^-) and mean LCI at baseline (patients in both groups, n=21).

4.5.2 Relationship between changes in NPD and lung function in response to treatment with gene therapy

Changes in NPD parameters (basal, dAMIL and total chloride response) from the active group were again correlated with changes in FEV_1 and LCI, in response to treatment, and are presented below.

One subject in the active group (LON-4087) was unable to perform spirometry at follow-up owing to a recent pneumothorax, therefore was excluded from the FEV_1 response to treatment analysis.

4.5.2.1 Basal and dAmil NPD measurement with FEV_1 in response to active treatment

A correlation was made between the changes in mean basal PD with the mean change in $ppFEV_1$. In response to active treatment, no significant correlation was identified between the changes in basal PD with the changes in FEV_1 (r=0.17, p=0.57; n=14, Pearson correlation). Similarly no correlation between the changes in dAmil and the changes in $ppFEV_1$were identified following active treatment (r=-0.06, p=0.83; n=14, Pearson correlation).

4.5.2.2 NPD Total Chloride with FEV_1 in response to treatment

A correlation between the mean changes in tCl^- PD with the mean change in $ppFEV_1$ was made to address whether any electrophysiological improvement in CFTR function (as demonstrated by an increase in tCl^- response) in response to gene therapy accompanied improvements in $ppFEV_1$. No statistically significant correlation was identified between the changes in $ppFEV_1$ and the change in total NPD chloride efflux following treatment with gene therapy (r=0.15, p=0.62; n=14, Pearson correlation) (Figure 4.27); no change in the placebo cohort was seen (r=-0.29, p=0.58; n=6).

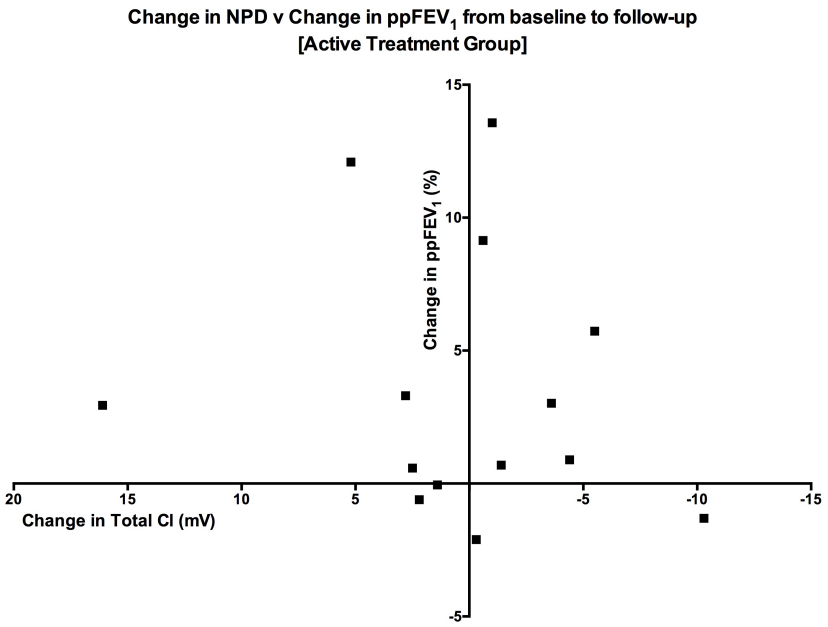


Figure 4.27. Correlations between change in nasal PD total chloride secretion and change in $ppFEV_1$ in response to treatment with gene therapy (n=14).

4.5.2.3 Changes in NPD parameters and LCI in response to gene therapy

For completeness, correlations were made between changes in NPD parameters and LCI, acknowledging upfront that no change was demonstrated in any of these indices in response to gene therapy.

Following treatment with gene therapy, no significant or clinically relevant correlation was measured between the changes in basal NPD with the change in LCI (r=-0.20, p=0.49; n=15, Pearson correlation). As no changes in dAmil were identified, no further correlations of sodium transport with LCI were made.

Finally a correlation was made between the mean changes in tCl^- NPD with the mean change in LCI, with changes in tCl^- and LCI demonstrating no correlation (r=0.10; p=0.72, Pearson correlation) (Figure 4.28).

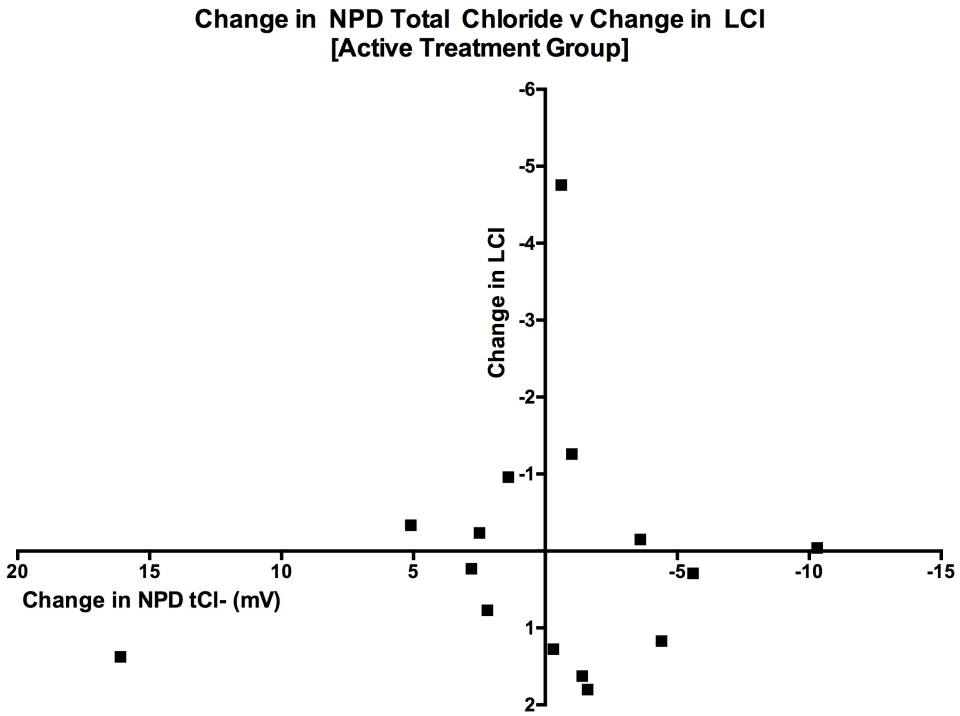


Figure 4.28. Correlation graph displaying change in NPD total chloride (tCl^-) and change in LCI in response to active treatment (n=15).

4.6 Validation of performing NPD blinded with 2 operators

NPDs performed pre-dosing and at follow-up were performed following a standard protocol, with the operator interacting with the trace. To prevent bias, as has previously been discussed (Chapter 3, section 3.2.12), NPDs performed 'on treatment' (after doses 3, 6 and 9) were all performed blinded to the operator, which required a second operator to 'be the eyes' of the NPD to feedback on the stability of the tracing and monitor for system errors, such as loss of electrical contact.

As this method had not been previously described, it was suggested by the author that validation of the technique, to provide confirmation of the MDT NPD results and for future research trials of CFTR-modulation, would be beneficial.

In total 4 second operators were trained, each being taught the NPD perfusion protocol, importance of trace stability and when to change perfusion solution; they each observed several NPDs being performed and reviewed NPD traces with the author (or KH) to identify and understand key points and common errors experienced during the procedure.

A post hoc validation was carried out to assess the suitability of this 2-person ('blinded') technique, by looking at the quality of the blinded NPD traces. This was achieved by (1) multi-observer quality assessment of a proportion of MDT NPD traces; (2) measurement of duration of NPD phases; (3) calculation of the proportion of NPD traces excluded from the final MDT analysis.

4.6.1 Methods used to validate blinded NPD technique

4.6.1.1 Multi-observer assessment of NPD quality

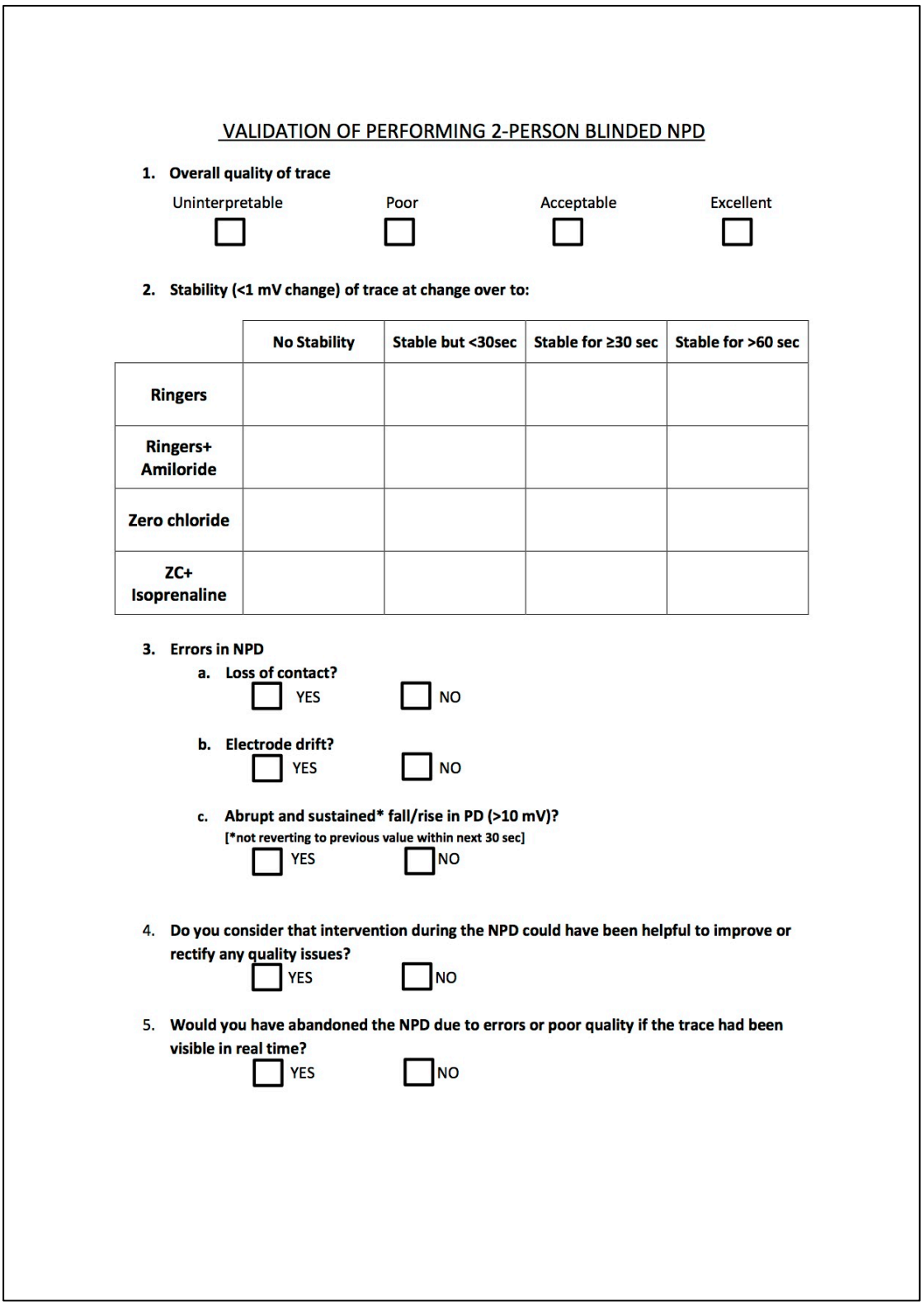
In total, 24 NPD traces (from all performed on the MDT) were randomly selected using online randomisation software – 12 traces were pre-dosing, thus being

performed by the standard NPD technique, by a solo operator (MDW or KH); 12 traces were performed blinded (either following doses 3, 6 or 9). All 24 traces were printed and randomly allocated an identification number, blinding all traces to the NPD method used.

The traces were independently given to 5 clinicians (MDW, KH, JCD, EA, NS), each familiar with both performing and analysing NPD, and asked to complete a score sheet for every trace (Figure 4.29). Using either a Likert scale or a binary (yes/no) response, the scores assessed the following quality of each NPD trace:

- Overall quality
- Stability at changeover (of solution)
- Electrical artefact (e.g. loss of contact, fall in PD)
- Would seeing the trace have made the operator intervene (e.g. re-fix the catheter or prematurely terminate the protocol)?

The responses were collated before the author was unblinded and the data grouped into the method by which the NPD was performed (standard or two-operator).



VALIDATION OF PERFORMING 2-PERSON BLINDED NPD

1. **Overall quality of trace**

Uninterpretable ☐ Poor ☐ Acceptable ☐ Excellent ☐

2. **Stability (<1 mV change) of trace at change over to:**

	No Stability	Stable but <30sec	Stable for ≥30 sec	Stable for >60 sec
Ringers				
Ringers+ Amiloride				
Zero chloride				
ZC+ Isoprenaline				

3. **Errors in NPD**

   a. **Loss of contact?** ☐ YES ☐ NO

   b. **Electrode drift?** ☐ YES ☐ NO

   c. **Abrupt and sustained* fall/rise in PD (>10 mV)?**
   [*not reverting to previous value within next 30 sec]
   ☐ YES ☐ NO

4. **Do you consider that intervention during the NPD could have been helpful to improve or rectify any quality issues?** ☐ YES ☐ NO

5. **Would you have abandoned the NPD due to errors or poor quality if the trace had been visible in real time?** ☐ YES ☐ NO

Figure 4.29. Score sheet used to grade quality, stability and artefact of NPD traces by each observer ("scorer").

4.6.1.2 Assessment of duration of the NPD phases

From the 4 solutions perfused during a NPD, the duration of perfusion of only 2 are subject to observer discretion – Ringer's and Ringer's/amiloride (RA), being changed when stability had been achieved for 30 sec (both zero chloride solutions have a fixed 5 min protocol). The author questioned whether the second observer would have a different level of confidence than a more experienced NPD practitioner, by demonstrating less certainty in when to change between perfusate, thus prolonging these two phases.

To calculate the duration of the RA phase, the author reviewed all MDT NPD traces downloaded into Microsoft Excel and measured the time between the electrical trace markers to signify the start of RA and start of ZCA.

As there is no electrical trace marker to signify the start of the Ringer's phase, the author had to interact with the trace and identify the typical change in shape in PD that occurs following nasal catheter insertion and the initiation of Ringer's perfusion. The estimated duration of Ringer's perfusion was calculated from this point until the electrical RA mark.

4.6.1.3 Exclusion of poor quality NPD traces

The total number of NPD traces that were excluded from all those performed on the MDT (pre-doing, on treatment, and at follow-up), was calculated, along with the reason for withdrawal of the trace – either for poor quality/not interpretable, for being a flat PD line; or other.

4.6.2 Results of independent observer assessment of quality and trace stability

All 5 clinicians ("scorers") independently scored all 24 traces; only 27 (4.5%) of responses for overall NPD quality and stability were unanswered, which includes one scorer who provided no response for stability for any ZCAI phase. On several

occasions, scorers would indicate a range between two Likert responses (e.g. between excellent and acceptable).

To allow interpretation of the data, a numerical score was assigned to each grade of quality (1 – 4) and stability (1 – 3), with responses answered in a range assigned a half integer value (e.g. 3.5); it was not the author's intention that the value or compound of score would reflect the magnitude of each NPD domain.

4.6.2.1 Assessment of NPD quality

All scorers' grades for each trace were together displayed graphically (Figure 4.30). On visual inspection, there was good concordance between the grading of NPD quality between scorers, other than one scorer (labelled scorer 'E') who, in general, rated the quality of traces much lower than other scorers ('A to D').

To determine whether there was an observed difference in quality between performing NPD blinded compared with the standard method, as assessed by blinded quality interpretation of these 24 traces, the median quality 'measurement' for each trace from all combined scorers was calculated (Figure 4.31).

In both NPD methods, the median quality of NPD trace was 'adequate' with no difference between the two methods being demonstrated. Other than all 5 scorers agreeing that one blinded trace was 'uninterpretable', these data supported that the quality of NPD performed by 2-operators was not inferior to that performed by the standard technique.

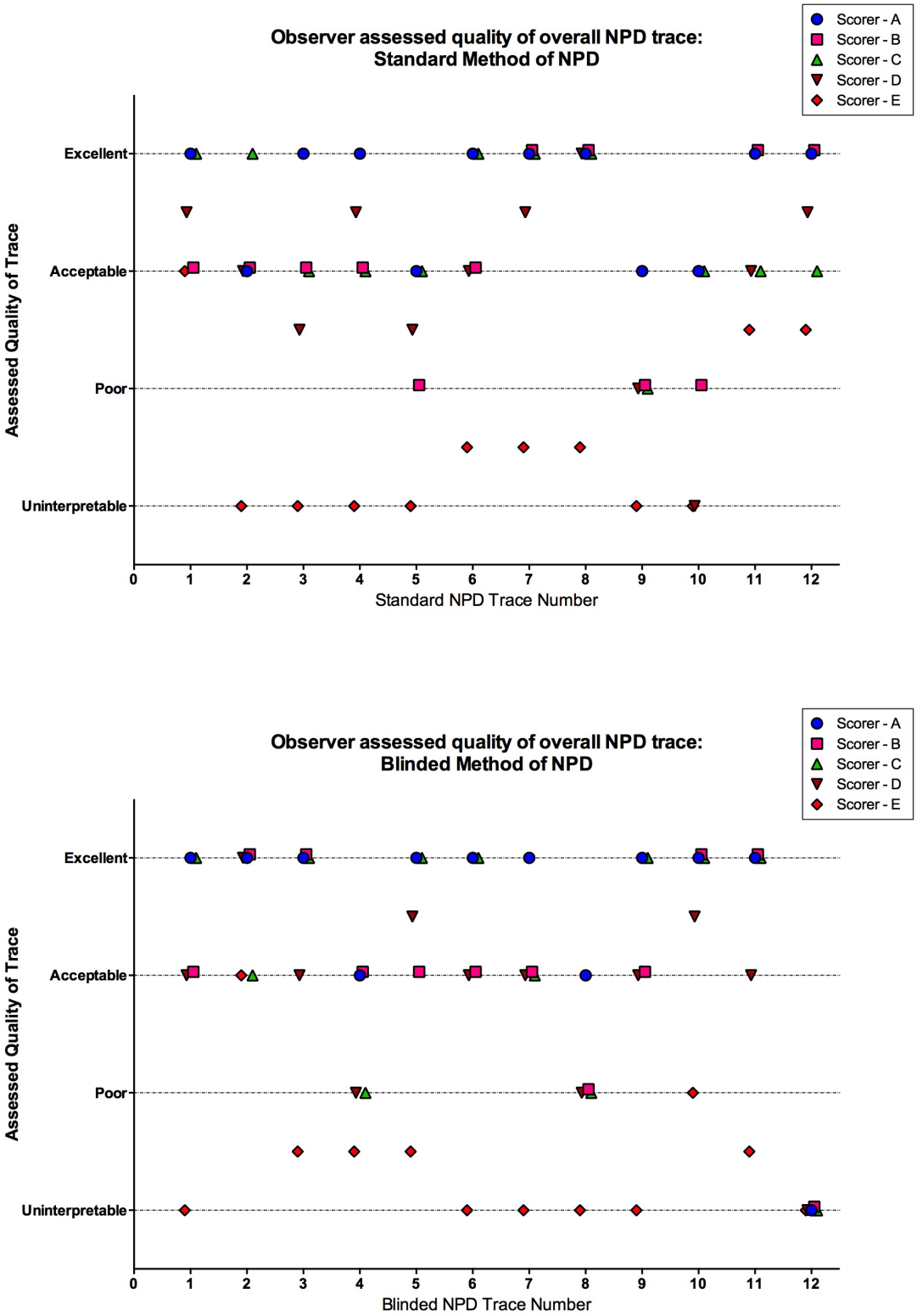


Figure 4.30. Combined plot of each independent observer's (scorer A to E) assessed quality of NPD traces performed (i) by standard method, n=12 (top); and (ii) by 2-operator (blinded) technique, n=12 (bottom).

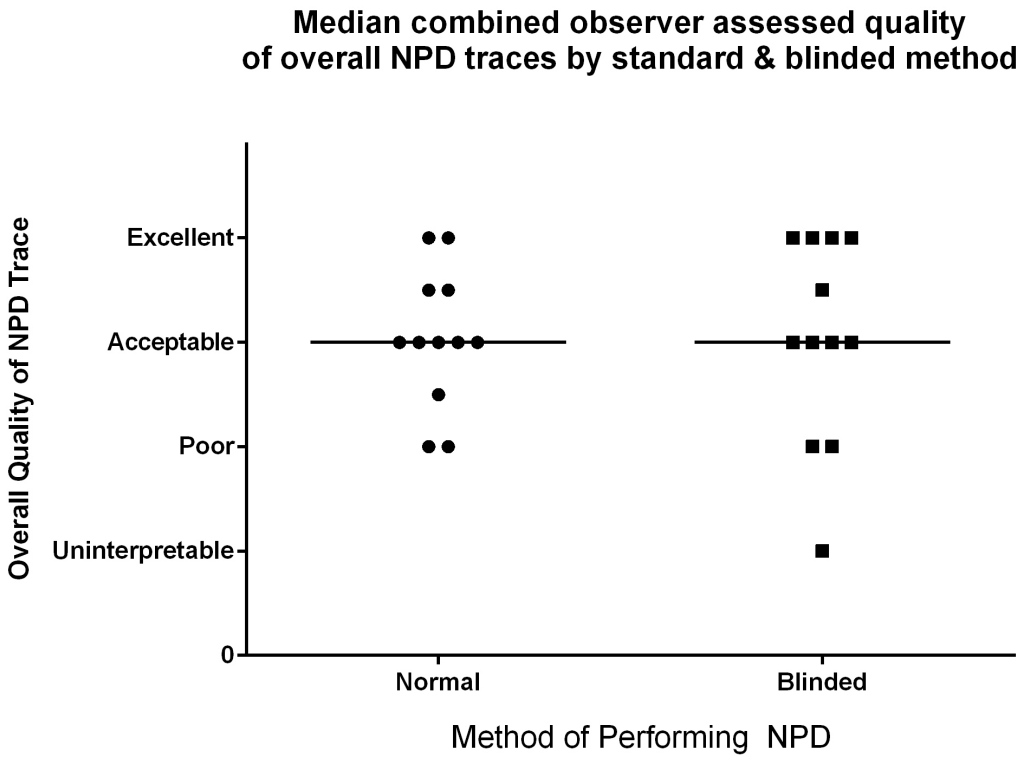


Figure 4.31. Median combined observer assessed quality of NPD traces as performed by standard (normal) and 2-operator (blinded) techniques (p=0.78 (Mann-Whitney U test)).

4.6.2.2 Assessment of blinded NPD stability

Using a similar method of numerical conversion of Likert responses for the stability of each phase of the NPD trace, individual scorer assessment of stability prior to change of perfusing solution was analysed and is plotted for 2 NPD phases: (i) Ringer's perfusion (Figure 4.32), (ii) and RA perfusion (Figures 4.33).

Stability of >30s is the ECFS standard for NPD electrical stability in Ringers and RA phases of the measurement, and hence shorter duration stability was assessed.

As stability is not required for the completion of ZCA and ZCAI phases, this was not analysed.

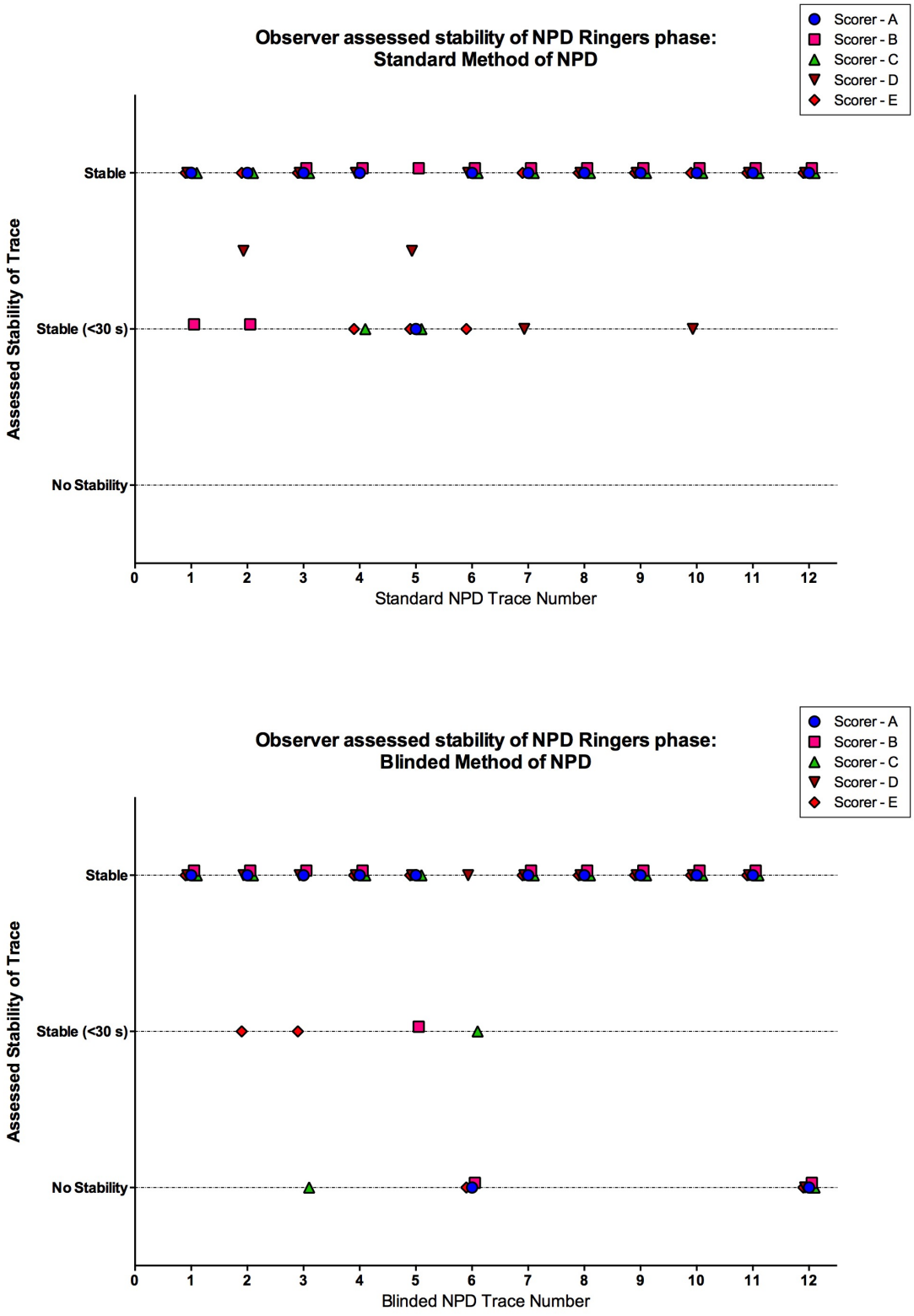


Figure 4.32. Combined plot of each independent observer's (scorer A to E) assessed stability of NPD traces following Ringers perfusion (i) by standard method, n=12 (top); and (ii) by a 2-operator (blinded) technique, n=12 (bottom).

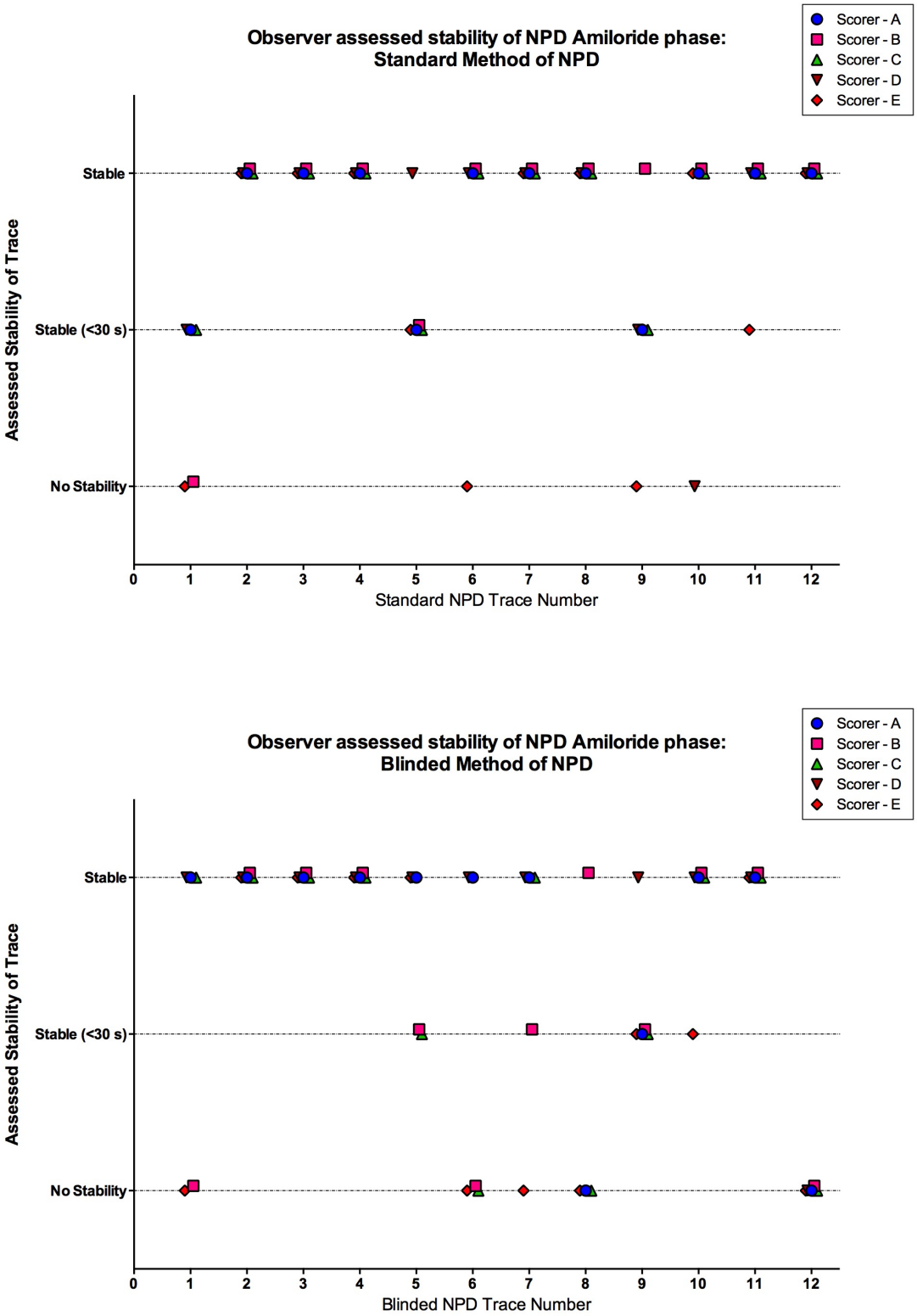


Figure 4.33. Combined plot of each independent observer's (scorer A to E) assessed stability of NPD traces following Ringers/amiloride perfusion (i) by standard method, n=12 (top); and (ii) by a 2-operator (blinded) technique, n=12 (bottom).

To summarise these data, the median quality from all observers' assessment for both NPD phases by both standard and 2-operator (blinded) method are displayed in Figure 4.34, which demonstrates no statistical difference between either method for both phases (Ringers: p=0.48; RA: p=0.44) – although it is clear that more blinded traces were unstable in both groups.

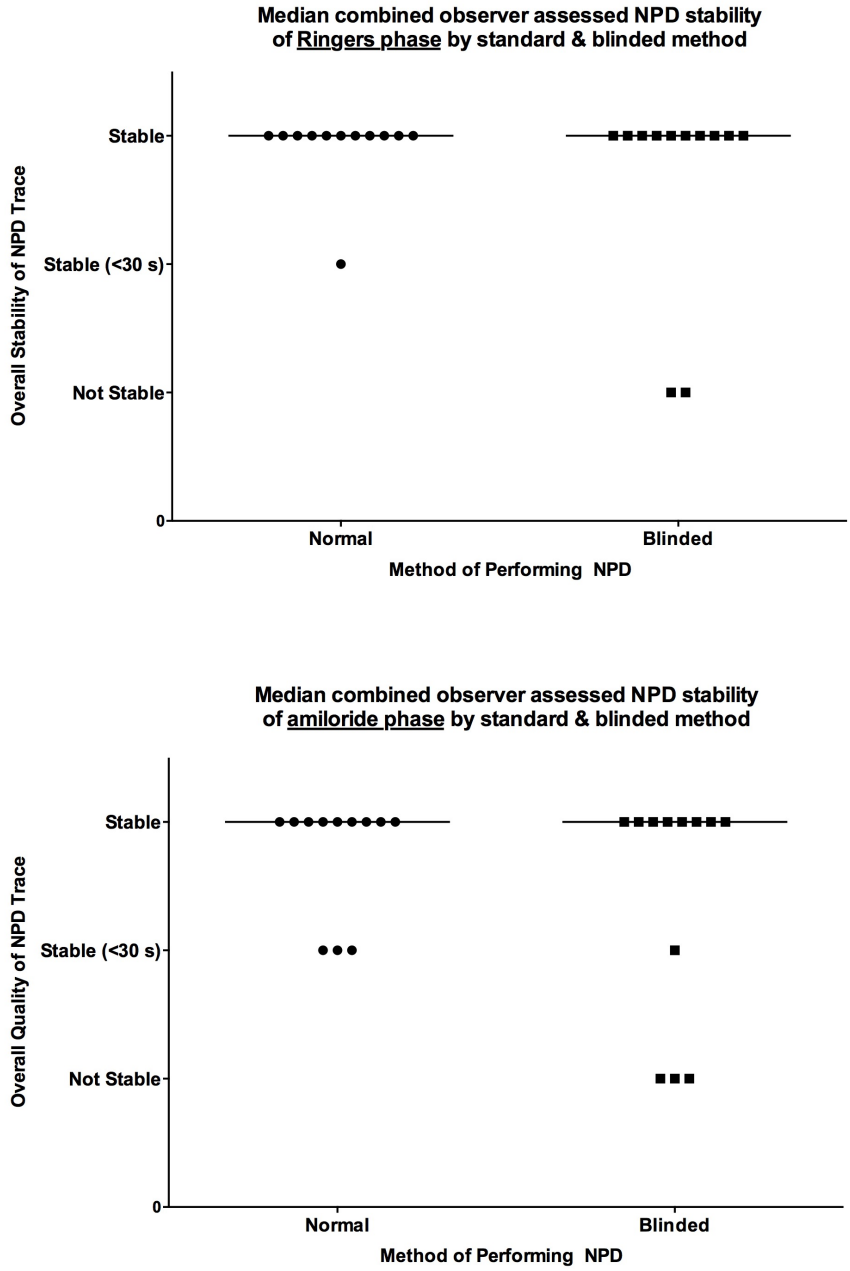


Figure 4.34. Median combined observer assessed stability of NPD traces following (i) TOP: Ringers, and (ii) BOTTOM: Ringers/amiloride perfusion, as performed by standard (normal) and 2-operator (blinded) techniques.

4.6.3 Duration of Ringer's and Ringers/amiloride perfusion

In total, 200 NPD traces were analysed (138 unblinded, 62 blinded). NPD traces were excluded when no discernible start point of the NPD phase was apparent or the phase was of poor quality – this is reflected in the difference in numbers of traces analysed.

There was no significant difference between the duration of the Ringer's or RA perfusion from all MDT NPDs performed by one operator or performed blinded with a second operator. The median estimated duration of the Ringer's perfusion of the NPD was 52 s longer when performed with a second operator, however this difference was not significant; the median duration of the RA perfusion was identical when performed by both methods. These data are summarised in Table 4.14 and Figure 4.35.

	Standard NPD Method	**2-operators NPD Method**	**p value** (Mann Witney U test)
Duration of Ringer's perfusion			
Number NPD Analysed	129	53	
Median (IQR), s	458 (343, 559)	510 (360, 638)	0.121
Duration of Ringers/amiloride perfusion			
Number NPD Analysed	133	52	
Median (IQR), s	399 (316, 531)	400 (326, 548)	0.719

Table 4.14. Summary of median (IQR) duration of Ringer's and Ringers/amiloride phases of NPD performed by standard or 2-operators (blinded) methods.

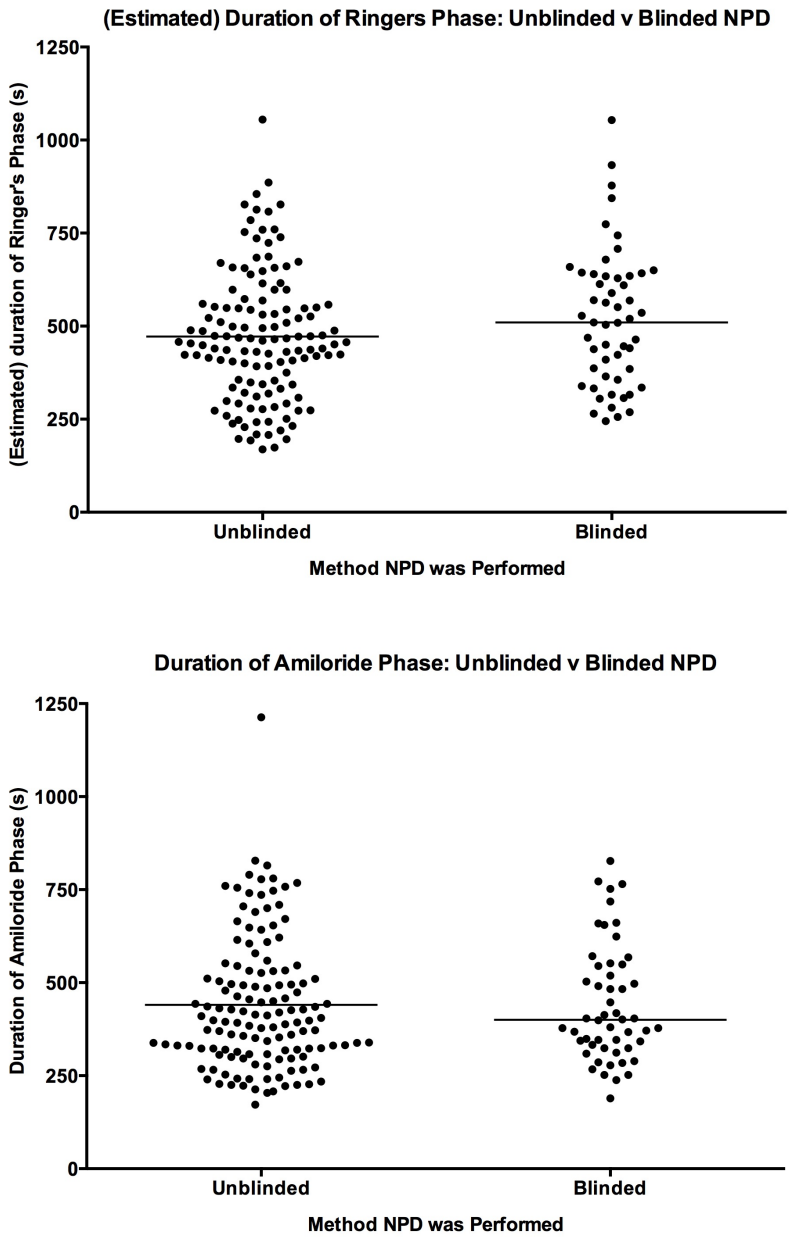


Figure 4.35. Individual (median) duration of (i) TOP: Ringer's, and (ii) BOTTOM: Ringer's/amiloride perfusion phases, of NPD performed by standard or 2-operator (blinded) methods.

4.6.4 Exclusion of NPD traces

A small proportion of NPD traces were excluded from the final analysis owing to poor quality. This raises the question of whether traces were more likely to be excluded if they were performed by the blinded technique, and whether the 5

'scorers' would have been more likely to intervene with or abandon the NPD during the blinded technique rather than the standard.

This has been addressed in two parts, firstly looking at the overall NPD traces within the MDT, and secondly using the 'scorer' response sheet to the 24 traces.

4.6.4.1 Traces excluded from MDT analysis

All traces performed during the MDT were used in this analysis – this included traces from patients who withdrew from the nasal subgroup prior to dosing. In total, 200 NPD traces were reviewed (138 performed by the standard NPD technique, 62 performed by a 2-operator blinded technique).

During their analysis of NPDs, MDW, JCD or both excluded a small proportion of traces from the MDT owing to poor quality, a flat/blunted PD response or when the PD trace became positive.

The total number of traces removed that were performed by the standard and blinded NPD methods for (i) poor quality, or (ii) any reasons, was analysed using the Fisher's exact test to answer whether a 2-operator technique produces an inferior quality of NPD trace. These data are summarised in Table 4.15.

	Standard NPD technique (n=138)	**2-operator (blinded) method (n=62)**
Traces Included, n (%)	128 (93)	54 (87)
All Traces Excluded, n (%)	10 (7)	8 (13)
Traces excluded (for poor quality), n (%)	3 (2)	6 (10)

Table 4.15. Table summary of number of NPD traces suitable for analysis or excluded as performed by standard (unblinded) or 2-person blinded methods.

There was no significant difference between the proportion of traces excluded for any reason between the two methods of performing a NPD (p=0.28, Fisher's exact test). Whilst the number of poor quality traces removed from analysis following both methods was small, a higher percentage of traces were excluded following 2-operator (blinded) NPD which was statistically significant (p=0.03, Fisher's exact test). Further analysis did not identify a statistically significant difference in the proportion of flat traces or other artefacts between the two methods.

4.6.4.2 Intervening or abandoning a blinded NPD

Finally, scorers were asked to record whether they would have intervened during the NPD (e.g. to either optimise the trace), or have abandoned the NPD measurement. In part this aimed to address whether amelioration of the NPD could have rescued a poor trace, and hence whether this opportunity was being missed when the NPD was performed blind.

The total number of NPDs in which the independent scorers would have intervened during the procedure was the same for both normal and blinded NPD methods (21 = 35%). In total, all scorers would have abandoned 3 (5%) traces by the standard NPD method, and 7 (12%) traces in the 2-operator (blinded) method, with no statistical difference being calculated between the two techniques (p=0.32, Fisher's exact test). The responses for all 12 traces for individual scorers/total for intervening and abandoning NPDs are shown in Figure 4.36.

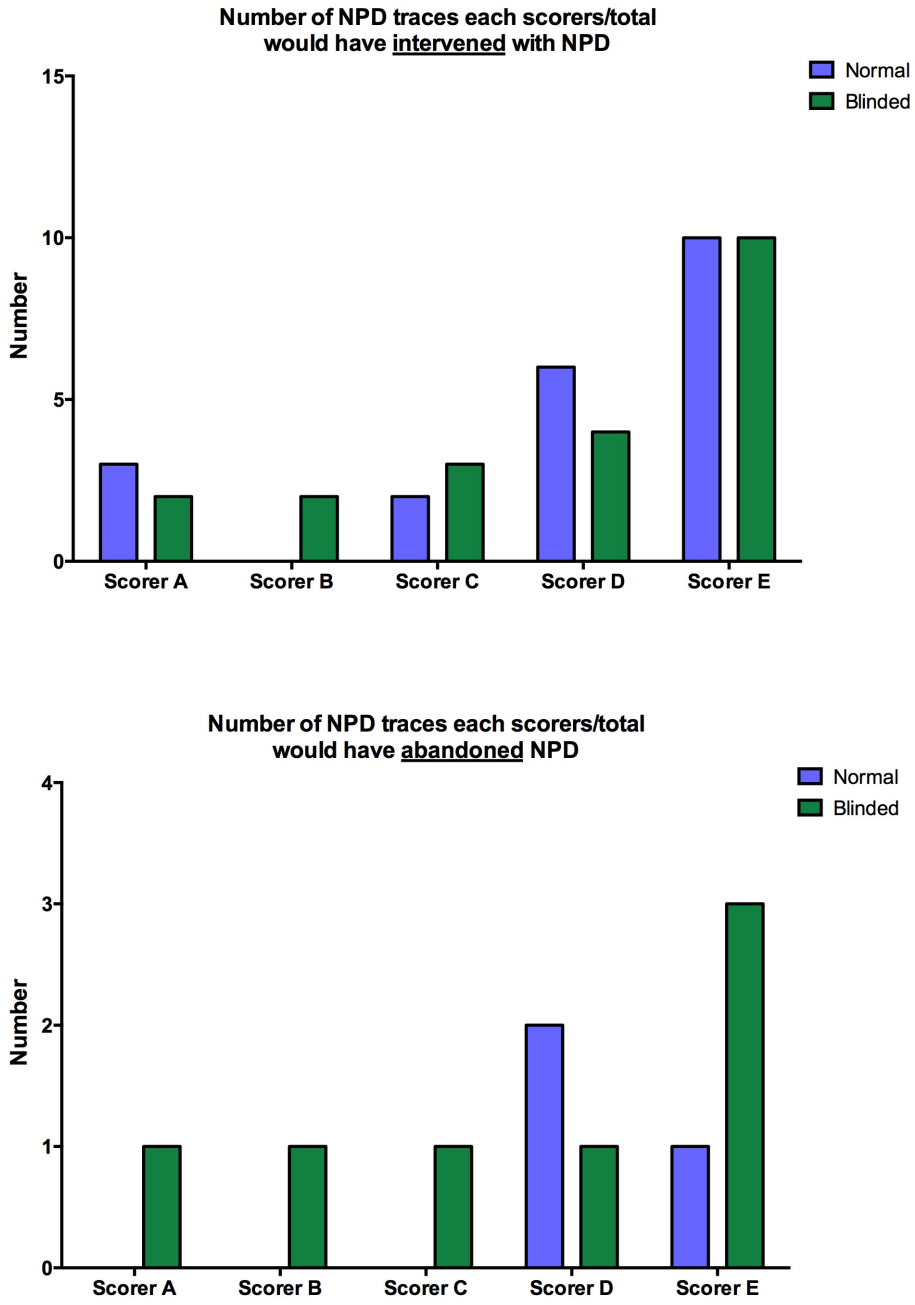


Figure 4.36. Bar graphs showing the total number of traces for each scorer/total traces, where scorer would have: (i) TOP – intervened with NPD; (ii) BOTTOM – abandoned NPD, as performed by standard and 2-operator (blinded) -methods.

4.7 Discussion

NPD is used as an electrophysiological assessment of CFTR function. This chapter has presented the electrophysiological outcomes of gene therapy on the nasal epithelium and possible definitions of response to treatment. It has reported the quantification of gene delivery to the nasal epithelium, and sought correlations between changes in NPD and changes in lung function. It has also considered a novel strategy for performing NPD using a 2-person technique to maintain blinding within a randomised controlled trial.

4.7.1 Change in upper airway electrophysiology in response to non-viral gene therapy

4.7.1.1 The effect of gene therapy on sodium transport

Assessment of CFTR function in response to gene therapy failed to identify changes in either sodium parameter, with no changes in basal or dAmil responses seen compared with the placebo group. As there is no 'threshold' to determine a treatment effect for the PD parameters, the author took changes greater than the CV for either parameter as a meaningful change. Using this method, 5 active and 2 placebo patients demonstrated depolarisation in both parameters at follow-up compared to baseline. The basal and dAmil responses of these patients from all NPDs measured at follow-up were analysed in more depth and nasal symptoms or an intercurrent URTI were found to be present at follow-up in 4/5 (active) and 1/2 (placebo) patients, suggesting that these changes appear to be related to inflammation of the nasal epithelium. Reduction in epithelial sodium ion transport due to inflammation is well recognised and is considered the explanation for these changes [214]. Figure 4.11 shows the variability in these patients' basal and dAmil measurements throughout the study, with changes being identified in both groups in the absence of nasal symptoms which makes these findings difficult to interpret.

It was not unexpected that changes in sodium transport did not change in response to active treatment, as this is in keeping with all the published gene therapy literature to date [148]. Indeed, even small molecule CFTR modulators have led either to no change in these parameters or to changes that are difficult to interpret (ataluren demonstrated no change in either parameter [215], and whilst ivacaftor led to a mean improvement of up to -14 mV following 14 days treatment in patients with *G551D*, no difference was demonstrated in a subsequent cohort after 28 days of treatment [127]). It has been suggested, as discussed in chapter 1, that to normalise the function of ENaC, close to 100% *CFTR* transfection is required, which current gene transfer is unable to achieve [156].

4.7.1.2 Changes in chloride transport after non-viral gene therapy

There was no significant difference in total chloride secretion between groups receiving treatment with non-viral gene therapy and placebo, but 4 subjects in the active group showed changes towards non-CF values. Firstly, the study was underpowered to detect significance in NPD parameters; secondly, this finding is in keeping with previous studies reporting variable responses (Chapter 1, section 1.11.6). Whilst hyperpolarisation, in keeping with improvements in chloride transport, was seen in 4 subjects in response to 12 months of gene therapy, similar changes were also seen in 1 patient from the placebo group. Improvements in chloride secretion following placebo were reported in 15% of patients in the randomised trial against ataluren therapy [138], and measurements of CF patients from the MDT at baseline similarly showed PD hyperpolarisation following perfusion of chloride-free solutions.

From an opportunistic look at the 4 active patients recording a tCl^- response (Figure 4.17), it is not possible to report any consistent changes throughout the study in regards to sustained or incremental NPD changes from these limited data. A similar assessment identified no difference between tCl^- responses measured14 and 28 days following dosing.

Despite the study reporting changes in FEV_1, a downstream measure of CFTR function, direct electrophysiological assessment of CFTR function has failed to show a treatment effect with gene therapy. A few patients have however shown evidence of changes in their nasal epithelial PD suggesting successful gene transfer in some patients. It is essential to remember that electrophysiological outcomes were not statistically powered unlike FEV_1, and the numbers enrolled reflected a cohort investigated opportunistically.

As the nasal brushing samples confirmed gene transfer to the nasal epithelium with increases in vector-specific DNA being measured in all active patient samples at follow-up, it is suggested that the discrepancy between gene delivery and CFTR measurement could be explained by several possibilities. Firstly it is likely that gene transfer is patchy and ineffective, and that NPD measurements are missing transfected epithelium; this could possibly explain the variability in tCl^- responses seen within the same patient. Similarly, it is suggested that the jet of gene therapy when administered to the nasal mucosa could have transfected epithelium more distal in the nasal cavity than where the measuring NPD catheter was placed, meaning measurements were taken from a lesser-treated area. Finally, it is suggested that measuring NPD at 14 or 28 days following dosing is outside the window of maximal gene expression, and that NPDs performed closer to dosing may have demonstrated a larger tCl^- response, as was demonstrated in measurements taken in the Pilot Study and reported in Dr Gwyneth Davies' thesis [211].

The author would therefore suggest that in future trials, an appropriately powered cohort of patients could be enrolled into a nasal subgroup, that NPD could be taken at intervals initially closer to dosing, and that measurements could be taken from both treated nostrils (rather than one) to increase the sensitivity of data.

4.7.2 Relationship between NPD and measurements of lung function

Studies of relationships between airway electrophysiology and severity of lung disease are inconclusive, with some reporting statistical correlations between sodium transport and FEV_1 [97, 216], or between chloride transport (dZCAI) and FEV_1 [100] while others have contested these relationships [217, 218].

For this thesis, I wanted to understand whether changes in airway PD measurements at baseline and following gene therapy correlated with downstream measurement of CFTR function, in terms of lung function.

Correlations between baseline NPD parameters and FEV_1 demonstrated weak, and non-significant, trends between higher sodium indices and worse lower FEV_1; no significant correlation between chloride transport was identified. No responses were identified in response to treatment. These data could not support the assertion that improvements in $ppFEV_1$ were related to NPD measured amelioration of CFTR function. However, this reflects the fact that the numbers of active patients at follow-up were small, the nasal subgroup was underpowered, and the overall lack of change in the PD parameters. Furthermore, the nasal subgroup was a mixed population, in particular with regards to age, and the data have not been corrected for such cofounders.

Unexpected linear relationships, that were statistically significant, were found at baseline between mean LCI and mean basal PD and dAMIL response, which have not been previously reported. The author hypothesises that this relationship could reflect that LCI is a more sensitive index of small airway function than FEV_1 [219], and more sensitive for detection of a correlation with epithelial PD – this relationship would support that increased sodium hyperabsorption leads to worse lung disease. Whilst the population analysed was heterogeneous (with regards to age and disease severity), this finding supports previous literature citing relationships between sodium transport and the severity of airway disease. No relationship between LCI and chloride transport was seen. This

finding would support an opportunity for future larger studies assessing airway inflammation and disease severity.

4.7.3 Variability of nasal PD measurements

The variability of baseline NPDs was measured for basal and dAmil responses across the entire intention to treat population (n=30). Basal PD measurements had a mean *CV* of 13.1%, which was in keeping with the published literature (mean *CV* 8.9% for 3 nPDs performed on alternate days (n=9) [220]; mean *CV* 14% for 2 NPD performed on different days (n=25) [221]) and that measured by EA (15% - unpublished data, MD thesis); similarly those for dAmil were within recognised variability (mean *CV* 33% for 2 NPD performed on different days (n=25) [221]). No post treatment *CV* was measured, but fluctuations in basal measurements over 12 months were seen in the placebo group (Figure 4.11, *f* & *g*).

It is not possible to measure *CV* from chloride responses in CF, owing to the denominator in the equation being close to zero, which leads to massive amplification of any observed variability. Seven patients demonstrated a chloride response above the non-CF threshold (-5 mV) on a single occasion (Figure 4.8), however owing to variability in their other measurements a mean tCl^- response fell within the CF range. This demonstrates variability, from the patient's physiology and the technique, within NPD measurements in the same individual, and when single measurements are used as an outcome measure, consideration of this should be taken. For this reason, the author would support that repeated post-treatment measurements be made on the same day in any future trials, to reduce the possibility of missing or over-reporting PD responses.

4.7.4 Assessing NPD response

There is no gold standard method for analysing NPD traces nor for quantifying an NPD chloride response. This chapter has outlined different methods for both, and presented their results, which will now be discussed.

4.7.4.1 Variation in methods used to analyse NPD results

This chapter has used two methods of analysing NPD: (i) a traditional method, using printed NPD traces, initially triaging and excluding any traces based on their quality, before then measuring PD. This approach allows the observer to interact with the trace, to measure PD and its changes. The author would propose that this method benefits from allowing the user's experience and understanding of epithelial electrophysiology to interact with the trace, however carries a risk of reducing standardisation and objectivity; (ii) an alternative method, analysing PD traces on an Excel spreadsheet, which is more pragmatic and could be standardised for multiple investigators to use. The capturing of electronic data allows PD to be measured to one decimal place, which may be important if small changes in PD are being measured. Nonetheless, it remains that an almost identical result the NPD response to total chloride was reported by both methods (Figure 4.18).

NPD interpretation is not included in the NPD SOP from either the ECFS or the CF Foundation Therapeutics Development Network (USA). It remains uncertain which measurement is best to report – the mean or most negative value, both having pros and cons in terms of reducing intra-subject variability and recording the largest signal of CFTR function; furthermore, it is unclear whether these measurements should be made from one nostril or from both. An ECSF NPD validation study has been recently completed (at the time of writing) and is anticipated to help address this uncertainty.

4.7.4.2 Defining an NPD chloride response

There is no standardised designation for defining a chloride response in clinical trials; although changes towards and into the non-CF range are desirable, the degree of improvement in hyperpolarisation and its correlation with clinical benefit remains unclear [204].

The author attempted to identify 'true' responders from treatment with gene therapy over those displaying background variation in the placebo group (section 4.3.3/Table 4.13). He used methods applied to NPD data from the Pilot Study by Dr. Gwyneth Davies for her thesis [211] – none of these subjects were treated with placebo and whilst varied responses were seen at different time points, it is impossible to ascertain the background "response" reflecting variation in NPD rather than optimised CFTR function.

When measuring any value greater at follow-up compared with any value at baseline, any pattern that is seen in both treatment arms likely reflects the variability of the assay. Furthermore, when measuring a change of greater than -5 mV from mean baseline to mean follow-up, similar proportions of patients are seen to 'respond' in both treatment groups, questioning the usefulness of this method. Finally, the author chose to add two equations specifically designed and validated for diagnostic use in CF and not as a trial outcome measure [212, 213] – neither equation was useful in identifying gene therapy responders. Both equations combine changes in chloride and sodium, with the latter not demonstrating a statistically significant change in the MDT in response to treatment, and therefore would not be an optimal index for defining a treatment response.

As they stand, the current methods are insufficiently reliable at identifying treatment responses. It is suggested that future work could consider modelling of chloride responses at different 'thresholds' to refine and delineate treatment responses.

4.7.5 CF patients demonstrating residual chloride secretion

Five patients were excluded from the nasal subgroup for having a mean baseline total chloride efflux significantly greater than -5 mV (approximately -10 to -15 mV response), with most of the chloride efflux occurring during ZCA phase (passive chloride transport) in 4 from 5 subjects. It is important to state that one patient was excluded on the basis of only one measurement being taken (-13.6 mV); all other patients had at least 2.

It is recognised that some patients with a milder CF phenotype can have residual CFTR function [222], and it is therefore probable that this could be demonstrated by a retained tCl^- response on NPD. These 5 patients could all be classified as 'mild' based on age of diagnosis, preserved lung and pancreatic function, infrequent exacerbations, and longevity; it is therefore not surprising that they each demonstrated a significant tCl^- response. From these patients, four had a *Phe508del* mutation on one allele, with the second being a gating (class III), class V mutation or being unclassified; one patient was homozygous *Phe508del* and was diagnosed aged 30 yrs, which is remarkably unusual and may suggest factors (genetic or environmental) other the *CFTR* mutation have a contributing role in disease phenotype [223].

The author feels that excluding patients with a partial tCl^- response was correct, and would support a similar triage in future studies with NPD as a trial outcome. A previous phase II study assessing changes in CFTR trafficking with miglustat failed to meet its primary outcome (NPD), citing that a significant proportion of patients had residual chloride secretion at baseline and that this limited a significant treatment effect [224].

4.7.6 Validation of performing NPD by a blinded technique

The results of this study showed that from a random selection of traces scored by 5 independent observers, when NPD was performed blinded (by 2-operators),

the quality of traces produced was not inferior to those performed by the standard method; however when comparing the entire collection of NPDs taken during the MDT, almost twice as many traces were excluded from the blinded compared to the standard data, with most being excluded for poor quality. It was also apparent from the analysis that the independent scorers would have abandoned the NPD more often in the blinded group, but the sample size was small (n=12). Furthermore, stability of the PD traces was slightly better at changeover when performed by the standard technique, and Ringer's perfusion was almost 1 min longer when performed by 2-operators (not significant), which perhaps reflects some hesitancy of the second operator to 'call' changeover.

It can be concluded that a 2-operator (blinded) technique did not impair the quality of the NPDs entering the final analysis of the trial, and served as a useful and practical solution to how to make on-treatment measurements; whilst a 2-operator, blinded method would be expected to take longer, and at the risk of rejecting more traces, this method remains a simple and valuable asset for groups where manpower or skills are rate-limiting.

The author would suggest that in repeating this method, he would ensure that fewer second observers were used to minimise inter-observer variability, and that each had more experience of the standard technique to increase their confidence to 'call' stability and perfusion changeover. The author feels there is an opportunity for mathematical modelling to create a computer-based system that would allow real-time NPD analysis in this blinded fashion. This system could remove human error and should incorporate measurements of trace stability and variability, to dictate the changeover of perfusate and announce errors in the system, allowing the NPD to be rectified or abandoned.

4.8 Conclusions

Whilst this chapter has shown some electrophysiological and molecular evidence of gene transfer in the nasal epithelium in response to *CFTR* gene therapy, the author must reject part (i) of the hypothesis that repeated nasal instillation of pGM169/GL67A can lead to a measurement improvement in nasal epithelial CFTR function as an overall treatment difference was not demonstrated; whilst the aims of this study were clear, this subgroup was underpowered. The results however have highlighted individual patients demonstrating mean NPD changes in a non-CF direction with a few patients approaching the non-CF range. It has been suggested that gene transfer to the epithelium is patchy and inefficient, which may explain discrepancies between vector-specific DNA and NPD-assessed CFTR function. Further explanations have been proposed, including NPD measurements being taken from an area of epithelium of reduced transfection, or at a time surpassing maximal gene expression.

The chapter has however demonstrated some clinically interesting correlations between baseline NPD measurements of sodium transport and measurements of lung function, especially the new finding of a relationship with LCI, thus accepting part (ii) of the hypothesis; these findings help to link the relationships between the severity of CF and sodium measurements that have previously been reported in other published studies.

Finally, this chapter has taken steps towards validating a 2-person NPD technique that could be adopted in situations where the research team was responsible for making NPD measurements and clinically assessing the patient and adverse outcomes.

Chapter 5: Lower Airway Potential Difference Measurement

This chapter will concentrate on the bronchoscopy subgroup of the Multidose Trial, initially focusing on the electrophysiological outcome measurements made of the lower airway at baseline and in response to treatment, and molecular measurements of gene transfer to the lower airway. It will report correlations tested between parameters of LAPD and lung function, similar to those made with the upper airway epithelium (chapter 4) but now focusing on measurements within the same organ. Finally it will explore a relationship between the upper and lower airway epithelium at baseline and in response to gene therapy.

The upper airway is frequently used as a surrogate for the lower airway, given its similar cellular properties and the fact that it is easily accessible. Little however is known about the true relationship between these CF epithelia at baseline, and their responses to gene therapy. It is of interest to know whether high basal PD measurements are seen in the same individual and both airway sites, and whether any residual chloride secretion in the nose or lower airway is similarly seen at the other site. To gain some understanding of the relationship between the behaviour of the nasal and lower airway epithelium in a CF population, correlations will be reported between the NPD and LAPD measurements from the MDT subgroups.

The hypothesis of this chapter is that non-viral *CFTR* gene therapy, nebulised at monthly intervals, will (i) demonstrate electrophysiological changes of *de novo* CFTR function, and (ii) that correlations will exist between LAPD measurements of ion transport and measurements of downstream CFTR function (i.e. lung function.

The methods used within this chapter have been described in chapter 3.

5.1 Multidose Trial – Bronchoscopy Subgroup

Patients were enrolled into the bronchoscopy subgroup (n=24) with a randomisation in favour of gene therapy (active:placebo = 2:1). To be eligible for the subgroup, it was essential that patients had no exclusion criteria specific to bronchoscopy (Chapter 2, section 2.3.5.2) and were clinically stable at the time of the procedure (otherwise the procedure could be postponed).

Bronchoscopies were performed before dosing, and at follow-up (27 to 36 days following dose 12, but always after both follow-up visits had been completed). LAPD was performed as per the methods described in chapter 3.

5.1.1 Recruiting bronchoscopy patients for an additional NPD

12 subjects enrolled only into the bronchoscopy subgroup, with the commonest reasons for not volunteering for the nasal subgroup being the additional time commitments and the thought of NPD being an unpleasant procedure. The author suggested that additional (non-trial protocol) NPD measurements could be made on these patients to increase numbers to address this question. A substantial amendment to version 5 of the protocol was made on this basis and was approved by GTAC. Subsequently, any patient enrolled into the bronchoscopy subgroup only was requested to undergo a single NPD after completion of their bronchoscopy; it was decided to perform these during general anaesthesia so as to minimise patient discomfort and interference from coughing, etc. These patients did not receive any nasal gene therapy and therefore the NPD could be undertaken at either pre- or post-dosing bronchoscopy, depending on patient preference and operating theatre logistics. However, in the analysis, any NPD performed at follow-up has only been included from patients subsequently found to be randomised to placebo, excluding the possibility that nebulised gene therapy could have contaminated

the nasal epithelium; Therefore assessments of the baseline untreated epithelium were made on what will be referred to as an 'untreated population'.

5.1.2 Analysis of NPD traces

The data files from all LAPD traces were loaded onto a PC and file names changed by MDW for blinded analysis. All LAPD traces were analysed by KH and JCD in Excel calculating the PD values electronically, and using a similar agreement method to that used by the author for LAPD analysis; a further analysis of traces was made by an external consultant (Steve Smith) using printed NPD traces who independently verified the results. All LAPD traces were printed from Excel, and remained blinded to treatment arm and relationship to dosing, and scored by EA using the method discussed in the nasal PD chapter; LAPD traces were removed from the analysis when their quality was deemed uninterpretable.

5.1.3 LAPD trace indices

Every trace was 'scored' for the following absolute PD and change (delta value (d)) for the following indices:

- Basal PD – in response to a perfusion with Ringer's solution, performed at the proximal airway (carina: anterior, posterior, left and right) and a 5th generation bronchus.

- Zero chloride with isoprenaline – in response to 5 min perfusion with ZCI solution at a 5th generation bronchus.

5.2 Results: Bronchoscopy Subgroup and Baseline LAPDs

5.2.1 Bronchoscopy subgroup – enrolment

A total of 25 patients enrolled into the bronchoscopy subgroup; 2 patients were withdrawn prior to dosing (one having cultured *M. abscessus* before dosing and the other being clinically unstable following screening).

In total, 23 patients were defined as the intention to treat bronchoscopy subgroup and had at least one dose - the baseline demographics of these patients are tabulated in Table 5.1; columns have been added summarising the demographics of the overall Multidose population to allow for a comparison with this cohort.

	Placebo Group		Active Group	
	Bronchoscopy Subgroup (n=7)	**MDT ITT Population (n=60)**	**Bronchoscopy Subgroup (n=16)**	**MDT ITT Population (n=76)**
Sex, n (%)				
Male	4 (57)	31 (23)	6 (37.5)	38 (28)
Female	3 (43)	29 (21)	10 (62.5)	38 (28)
Age, yr, median (IQR)	33.0 (13.0, 42.0)	21.5 (15.0, 34.5)	21.5 (14.3, 27.8)	23.0 (16.0, 30.0)
By Age Group, n (%)				
Adults	5 (71)	19 (21.7)	12 (71)	25 (32.9)
Children (<16 yr)	2 (29)	41 (68.3)	5 (29)	51 (67.1)
$ppFEV_1$, mean (SD) (%)	67.0 (10.6)	67.9 (10.1)	71.1 (8.2)	69.2 (11.4)
LCI, median (IQR)	10.1 (9.1, 12.1)	10.9 (9.6, 12.8)	8.6 (8.0, 9.7)	9.9 (8.5, 11.8)
***CFTR* genotype, n (%)**				
F508del/F508del	4 (57)	-	10 (62.5)	-
F508del/other	3 (43)	-	6 (37.5)	-
Baseline LAPD, mean (SD) (mV)				
Proximal Basal	-20.9 (11.4)	-	-16.8 (5.2)	-
Distal Basal	-13.9 (3.2)	-	-15.2 (5.5)	-
Distal tCl^-	0.6 (2.7)	-	0.6 (3.2)	-

Table 5.1. Baseline (pre-dosing) demographics of the intention to treat patients in the bronchoscopy subgroup and entire Multidose population, for placebo and active treatment arms. The table includes genotype, assessments of lung function (FEV_1 and LCI) and mean group baseline LAPD measurements.

5.2.2 Bronchoscopy subgroup – completion of study

From the 23 patients entering the dosing phase of the trial, a total of 21 post-dosing bronchoscopies were performed – 14 active group; 7 placebo group. The following explains in detail reasons for a missed or delayed follow-up bronchoscopy, or the absence of post-treatment data.

5.2.2.1 Withdrawal from study: active treatment group

2 patients withdrew from the study (neither due to adverse medical symptoms):

(i) Patient LON-4065, having completed 6 doses, wished to exit the trial to discontinue contraception precautions, volunteered to have an exit bronchoscopy – her PD data have been included in analyses in this thesis, but correlations with follow-up FEV_1 and LCI data have been excluded, to allow comparison of subjects receiving ≥9 doses.

(ii) Patient LON-4073 withdrew from the trial after 9 doses, did not complete an exit bronchoscopy and therefore only their baseline data have been included.

5.2.2.2 Unwell at follow-up bronchoscopy: active treatment group

(i) Patient LON-4087 was clinically unwell at follow-up, having experienced an iatrogenic pneumothorax following a procedure to replace an indwelling venous access device and was considered clinically unfit for an elective procedure; trial bronchoscopy was therefore cancelled.

(ii) Two patients, both in the active group, had follow-up bronchoscopies delayed for medical reasons and performed outside of the protocol window: patient LON-4027 had acute pancreatitis and LON-4056 an acute pulmonary exacerbation; their bronchoscopies were performed at

111 and 49 days respectively. These patients have been excluded from all post-dosing PD analyses as it was felt that this exceeded the likely duration of gene expression (bronchial wall biopsy samples were taken as protocol and analysed as a safety measure – these results are not discussed within this thesis).

5.2.2.3 Incomplete LAPD measurements: active treatment group

In one patient (LON-4057), baseline PD measurements were unable to be taken due to technical failings; in a further patient (LON-4049), post-dosing chloride PD measurements were technically unable to be made.

In total, 10 matched pre/post-dosing basal, and 9 total chloride LAPD measurements were available from the active treatment group for analysis.

5.2.2.4 Delayed follow-up bronchoscopies: placebo group

All patients in the placebo arm completed bronchoscopies, with 2 subjects having their procedures outside the protocol window.

Patients LON-4031 and LON-4069 both had acute pulmonary exacerbations at follow-up, and therefore their bronchoscopies were performed at 58 and 62 days respectively. These patients are included in follow-up analyses, as it was felt unlikely that treatment with placebo would have altered any LAPD measurements.

In total, 7 patients have matched pre/post-treatment for all LAPD indices in the placebo group.

5.2.3 Bronchoscopy-related adverse events

Occasional, self-limiting adverse events were recorded in a minority of patients following bronchoscopy (sore throat, headache and fever), all of which resolved without medical intervention.

One patient (LON4060) experienced an episode of haemoptysis during the bronchoscopy following endobronchial brushing, which resolved spontaneously but prematurely terminated the procedure.

One serious adverse event (SAE) was recorded following a post-dosing bronchoscopy – patient LON4068 was discharged following the procedure but was later admitted to hospital with a new onset of pyrexia, headache and 'flu-like symptoms and subsequently developed a pulmonary exacerbation requiring intravenous antibiotics. This event was reviewed by the DSMB and considered possibly related to the bronchoscopy.

5.3 Lower Airway Potential Difference at Baseline

5.3.1 Baseline basal LAPD measurements

Basal LAPD was measured in response to Ringer's perfusion (i) proximally, at 4 sites around the carina (anterior, posterior, left and right) and (ii) distally, at 3 different (approximate) 5^{th} generation airways on the same lung, and a mean of the measurements taken.

Baseline LAPD measurements were not available from 1 patient (LON4057 – a child). Only 1 proximal basal PD could be recorded at baseline from patient LON4065, but a complete proximal data set was collected at follow-up.

5.3.1.1 Variation in basal measurements between age and airway site

A total of 72 proximal measurements and 58 distal measurements were taken and the mean values for each patient have been analysed. Whilst the adult proximal basal PD was approx 4 mV higher than those of in the children, there was no significant difference in the basal mean LAPD between adults and children at either the proximal (p=0.51, paired t-test) or distal (p=0.98, paired t-test) airways (Table 5.2). Distal basal PD measurements were lower (less negative) than the corresponding proximal basal measurements in the entire intention to treat group (p=0.32, paired t-test) and lower in an adult subgroup (p=0.27, paired t-test), in keeping with previously published data [208] – see Figure 5.1; there was no difference between basal measurements in children at different sites, although the small numbers limit interpretation in this group (p=0.93).

Figure 5.1 displays all patients' basal LAPD values at either site, with a mean value shown, and a separation between the site of the LAPD and the age (adult v child) of the patient.

	All Patients (n=24†)		**Adult (n=16)**		**Child (n=6†)**	
	Proximal	**Distal**	**Proximal**	**Distal**	**Proximal**	**Distal**
Basal LAPD Median (IQR), mV	-15.6 (-9.5, -23.2)	-12.3 (-8.7, -20.2)	-17.6 (-11.0,-23.6)	-12.2 (-8.7, -19.6)	-12.2 (-8.1, -22.8)	-12.4 (-7.9, -21.1)
p-value	0.03*		<0.01*		0.61	

Table 5.2. Table summarising median (IQR) basal LAPD measurements at baseline from the ITT bronchoscopy subgroup from all patients' basal measurements at the proximal and distal airway, and by adult/child separation. †One patient (child) had no basal measurements at baseline. Statistical analysis using the Wilcoxon paired rank test *p<0.05.

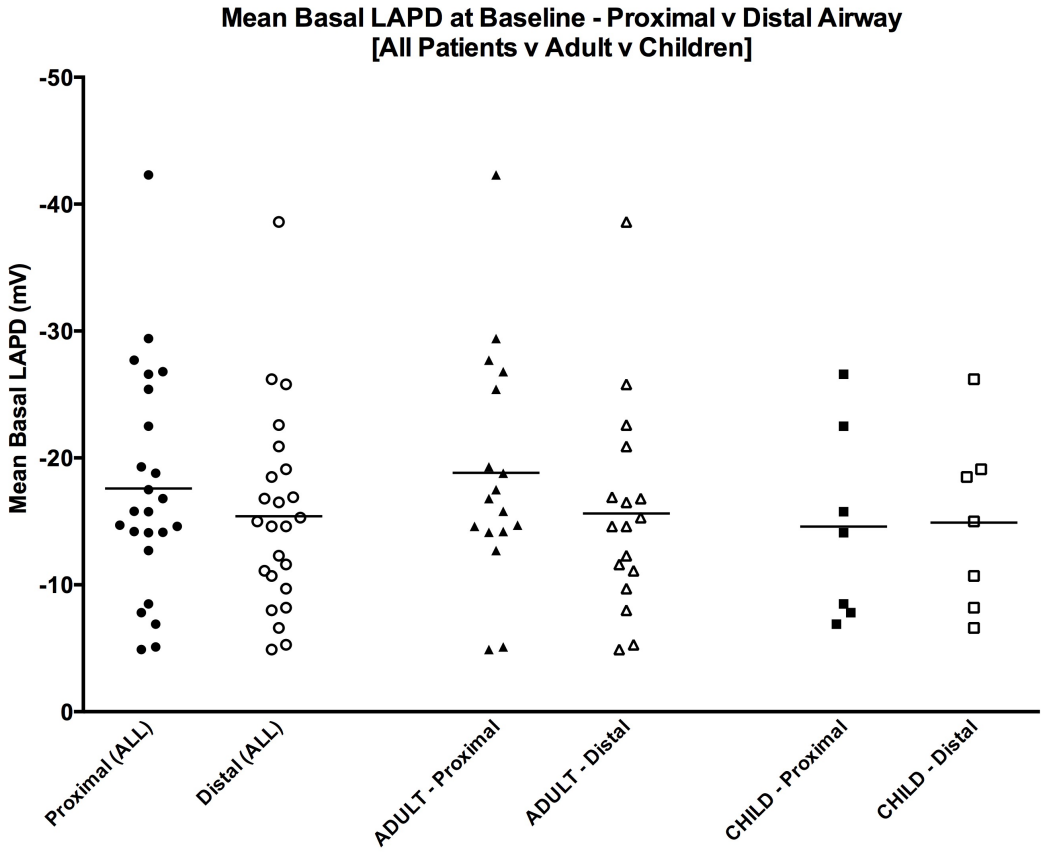


Figure 5.1. Graph of individual patient mean basal LAPD measurements (at baseline) from the ITT bronchoscopy subgroup, at proximal and distal airway. Comparisons of (i) all subgroup (ii) adults and children patients separated.

5.3.1.2 Most negative LAPD basal measurement versus site and age

To address whether taking all measurements from each individual had possibly diluted an effect of any difference between the groups and airway sites, a repeat analysis using the most negative basal measurement at both sites for each patient was performed. The results demonstrated that there was no difference between sites in the group as a whole, nor in the subgroups divided by age. The data of these analyses have been summarised as mean values in Table 5.3 with Figure 5.2 showing only the most negative LAPD basal measurement from each patient at either site; mean values for each representation have been presented.

	All Patients (n=24†)		Adult (n=16)		Child (n=6†)	
	Proximal	**Distal**	**Proximal**	**Distal**	**Proximal**	**Distal**
Most negative Basal LAPD Median (IQR), mV	-24.0 (-16.1, -30.4)	-20.6 (-12.2, -27.2)	-24.4 (-18.8, -31.4)	-20.5 (-12.1, -26.0)	-16.6 (-9.4, -28.6)	-23.0 (-13.3, -28.7)
p-value	0.35		0.14		0.69	

Table 5.3. Table summarising median (IQR) 'most negative' basal LAPD value at each site and by adult/child separation. †One patient (child) had no basal measurements at baseline. Statistical analysis using the Wilcoxon paired rank test *p<0.05.

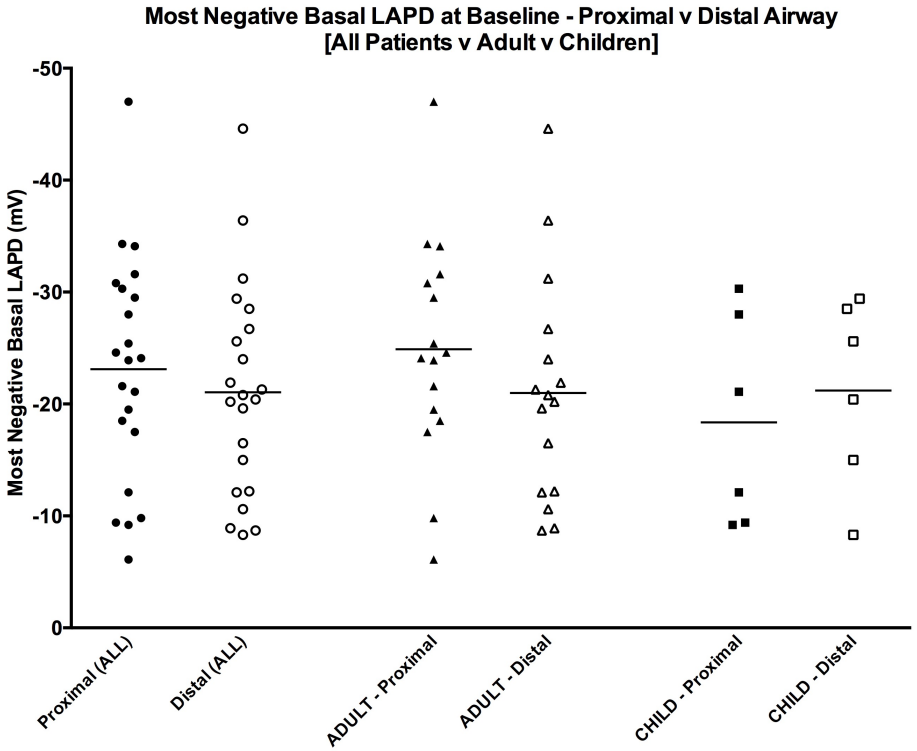


Figure 5.2. Graph of individual patient 'most negative' basal LAPD measurements (at baseline) from the ITT bronchoscopy subgroup, at proximal and distal airway. Graph shows comparisons of (i) all subgroup, (ii) adults, and (iii) children.

5.3.1.3 Correlation between proximal and distal basal LAPD (baseline)

To appreciate whether a relationship between the proximal and distal basal LAPD exists, a dot plot graph was constructed using (i) the baseline mean measurements (n=24), (ii) the most negative baseline basal measurement (n=24), and the correlation between the two variables measured. By either method of analysis, the proximal and distal basal LAPD did not correlate significantly: (i) mean basal LAPD: r=0.19, p=0.37 (Figure 5.3 a); (ii) most negative LAPD: r=0.18, p=0.41 (Pearson correlation coefficient) (Figure 5.3 b).

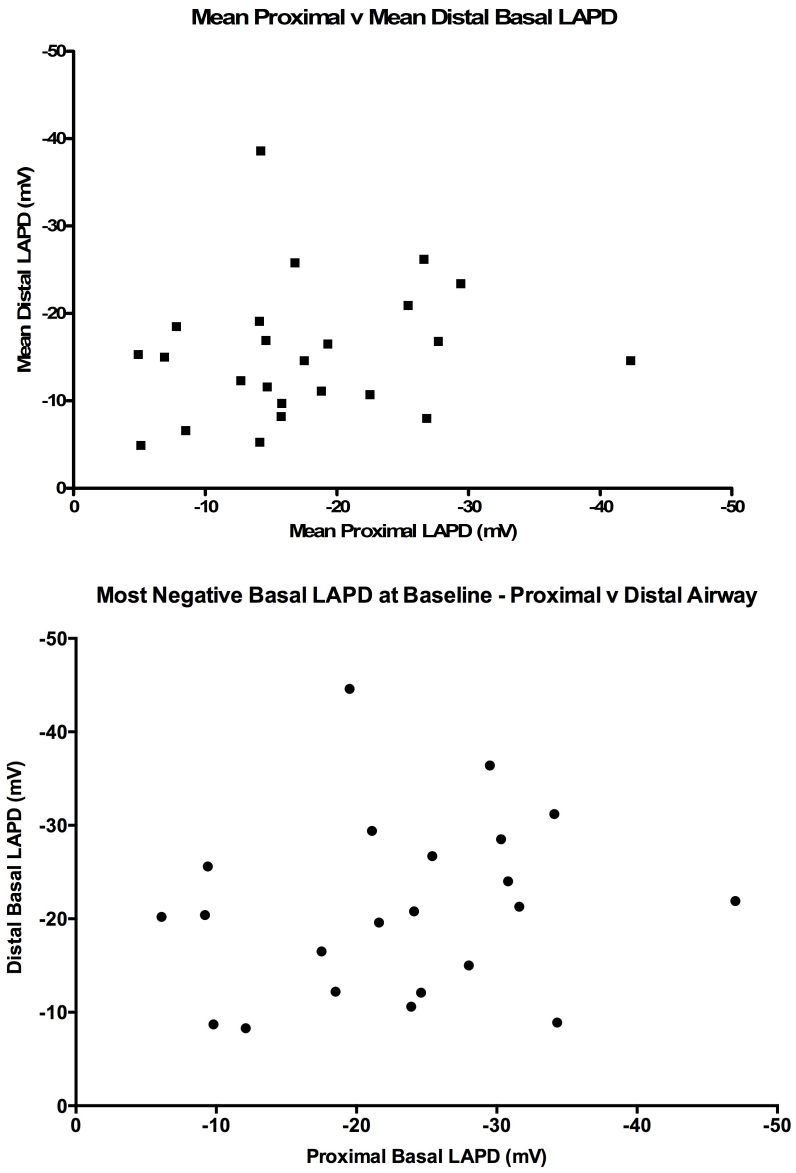


Figure 5.3. Graph of proximal basal against distal basal LAPD at baseline (n=24): (a) TOP: using mean basal values; (b) BOTTOM: using most negative basal values.

5.3.2 Baseline total chloride LAPD response

The total chloride response (tCl^-) was measured in response to 5 min perfusion of ZCI solution at the same 3 distal sites immediately following measurement of basal PD; measurements were not made at the proximal airway to prevent the distal draining and pooling of solutions, precluding measurements at this more important site. In 2 subjects, both in the active treatment group (one pre-dosing, the other at follow-up), no total chloride response could be obtained; in 5 separate cases, only 2 rather than 3 measurements were possible.

The mean (SD) baseline total chloride response from the ITT group was 0.7 mV (3.3) mV. Figure 5.4 summaries individual baseline tCl^- responses (mean and range) from the ITT bronchoscopy subgroup.

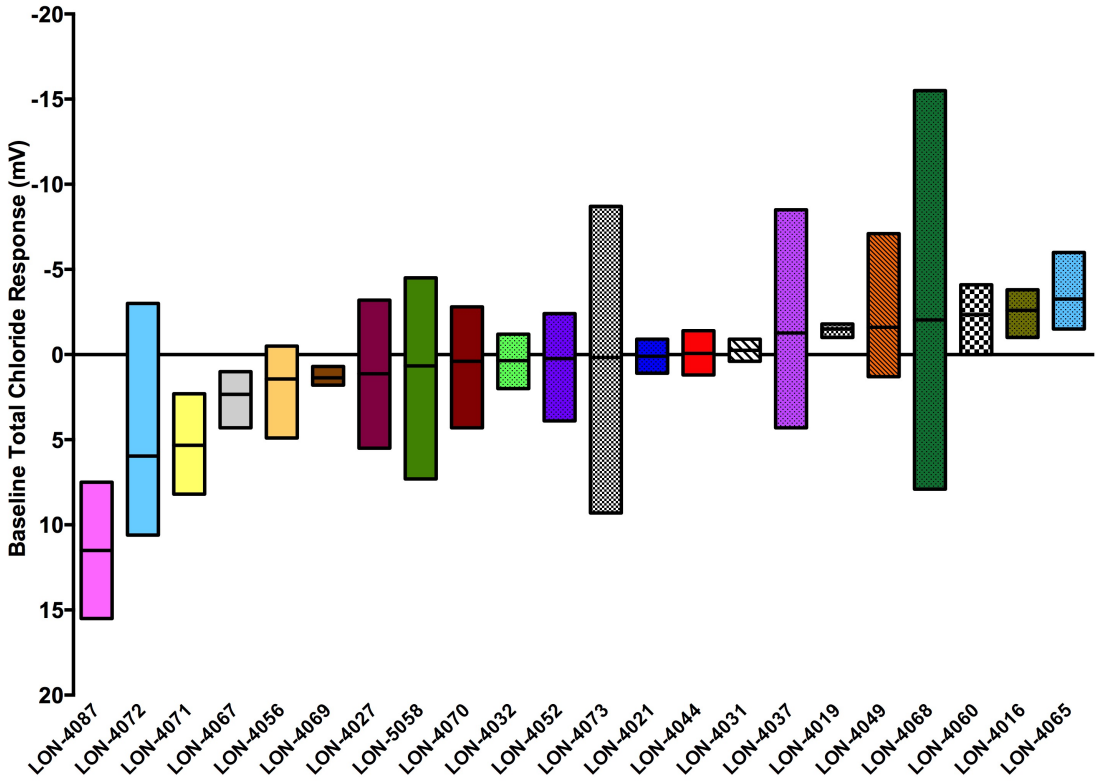


Figure 5.4. Graph of individual subjects' LAPD baseline tCl- responses (mean and range) from the ITT bronchoscopy subgroup, ordered by decreasing mean chloride response. (No exclusionary response was pre-defined and hence no red demarcation line is displayed on this graph as was seen in the NPD image.)

5.4 LAPD response to treatment with gene therapy

5.4.1 Change in basal LAPD in response to treatment

A total of 17 paired basal LAPDs were available for a response to treatment analysis (10 active; 7 placebo). There was no difference at baseline between the two treatment groups.

The mean (SD) proximal and distal LAPD results pre- and post- treatment from the two treatment groups is summarised in Table 5.4.

	Placebo Group (n=7)			**Active Group (n=10)**		
	Baseline	**Follow-up**	**Change**	**Baseline**	**Follow-up**	**Change**
Proximal Basal Mean (SD), mV	-20.9 (11.4)	-16.4 (4.9)	4.5 (11.3) p=0.33	-16.6 (6.4)	-14.7 (6.8)	0.7 (9.4) p=0.53
Distal Basal Mean (SD), mV	-13.9 (3.2)	-14.9 (5.1)	-1.0 (5.7) p=0.70	-16.3 (7.2)	-14.0 (6.6)	2.9 (9.7) p=0.51

Table 5.4. Table summarising mean (SD) proximal and distal basal LAPD response to treatment (active and placebo). Results of a paired t-test are shown.

There was no statistically significant change in response to treatment in basal LAPD at either proximal or distal epithelial sites in response to gene therapy, nor was there identified a trend towards a reduction (normalisation) in basal PD (Figure 5.5). The changes seen from baseline to follow-up in response to gene therapy did not differ significantly from those seen in the placebo group (proximal p=0.45, distal p=0.35, unpaired t-test).

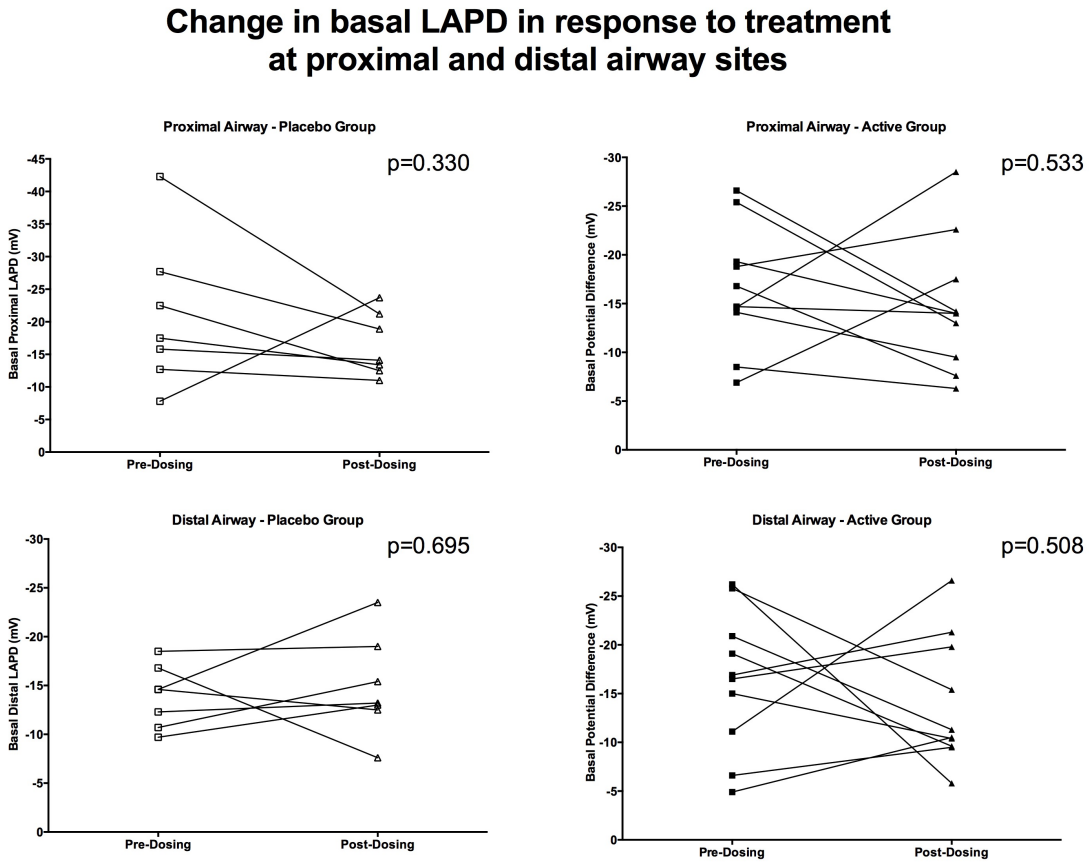


Figure 5.5. Change in (TOP) proximal and (BOTTOM) distal basal LAPD measurements in both (LEFT) placebo (n=7) and (RIGHT) active (n=10) treatment groups in response to treatment. Results of paired t-test are shown.

5.4.2 LAPD total chloride in response to treatment with gene therapy

Having excluded 2 patients (active group) for bronchoscopies performed outside the protocol window, and 2 patients where measurements were unable to be obtained, a total of 17 patients (10 active, 7 placebo) had paired pre-/post-dosing total chloride measurements analysed – this includes one subject (LON-4065) having measurements taken after 6 doses of gene therapy.

The mean (SD) total chloride responses pre- and post- dosing for both treatment groups are summarised in Table 5.5, and shown in Figure 5.6.

	Placebo Group (n=7)			Active Group (n=10)		
	Baseline	**Follow-up**	**Change**	**Baseline**	**Follow-up**	**Change**
tCl^- Response Median (IQR), mV	0.4 (1.5, -3.0)	1.4 (3.3, 0.3)	3.1 (5.3, -1.2) p=0.22	-0.2 (1.6, -2.1)	-0.5 (2.1, -5.5)	-1.3 (2.4, -3.6) p=0.49

Table 5.5. Table summarising mean (SD) LAPD total chloride response following treatment (active and placebo). (Wilcoxon paired rank test).

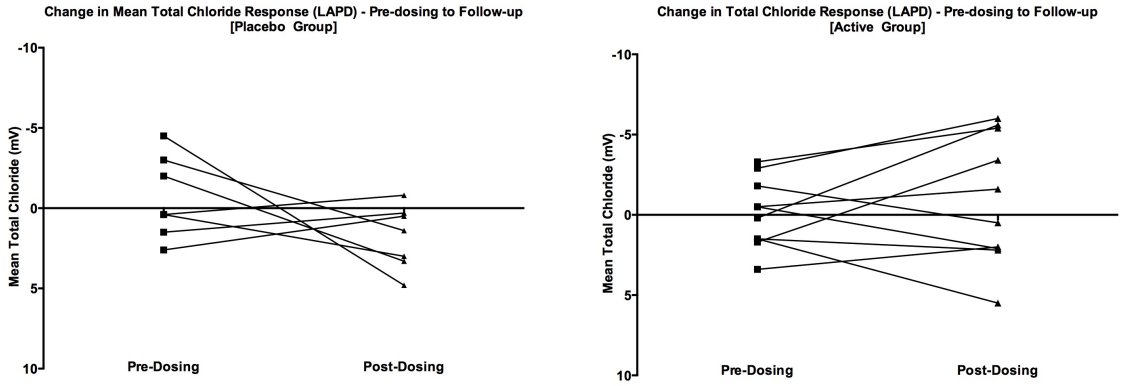


Figure 5.6. Change in LAPD total chloride response in both placebo (n=7) and active (n=10) groups in response to treatment.

From these data, a statistically significant treatment effect was demonstrated in response to gene therapy, with a treatment response in LAPD tCl^- of -4.4 mV to gene therapy compared to placebo (p=0.03, Wilcoxon paired test) (Figure 5.7).

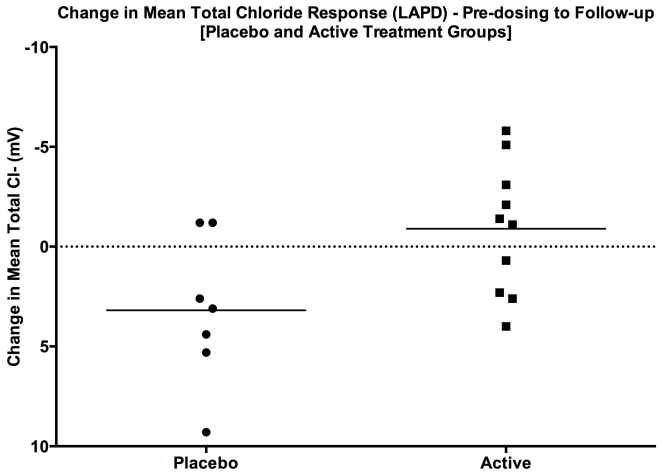


Figure 5.7. Graph summarising the changes in individual subject's mean tCl^- in response to placebo and active treatment, the mean group treatment, and the treatment difference (-4.4 mV, p=0.03, Wilcoxon paired test).

5.4.2.1 'Responders' with improved LAPD chloride efflux

In both randomisation groups, improvements (i.e. a more negative LAPD tCl^- response) were seen at follow-up in some subjects, supporting an increase in chloride transport through the distal lower airway epithelium.

In the active group, 5/10 (50%) patients treated with gene therapy had a mean (SD) improvement in tCl^- response of -3.9 mV (1.5), with 3 (30%) patients measuring a mean tCl^- response of between -5.4 to -6.0 mV at follow-up. 3/10 (30%) of patients measured an overall negative tCl^- response greater than any pre-dosing value (between -3.2 and -5.4 mV).

In the placebo group, 3/7 (43%) patients however also demonstrated a similar result, with a mean (SD) improvement in tCl^- response of -3.0 mV (0.4), however the mean tCl^- response of all patients at follow-up remained in the positive PD range, or close to 0 mV.

5.5 Quantification of Transgene *CFTR* cDNA and mRNA in the Bronchial Epithelium

Bronchial brushing samples were taken from all patients at pre-dosing and follow-up visits (placebo = 6, active = 14), excluding those that had withdrawn; 1 patient in the active group (LON-4060) had brushing terminated prematurely (after 5 from 10 brushings) due to significant bronchial bleeding. For the analysis, the two active patients having bronchoscopies outside the follow-up window have been included as an intention to treat analysis.

Vector-specific *CFTR* DNA and mRNA was quantified as previously described (Chapter 3, section 3.4.3). All patients (in both treatment groups) had no measured DNA at baseline. At follow-up, a statistical difference following delivery of gene therapy to the lower airway was seen, with 12/14 (85.7%) patients demonstrating increases in cDNA ($p<0.01$); the other 2 patients (14.3%) had no vector-specific DNA measured at follow – one of these two patients was LON-4027 whose bronchoscopy was 111 days following their last dose of gene therapy (Figure 5.8). In the placebo group, one (16.7%) patient had bronchial DNA reported as PBNQ, with all other placebo patients having no DNA measured (83.3%).

No vector-specific mRNA was measured at baseline or at follow-up from any patient in either treatment group.

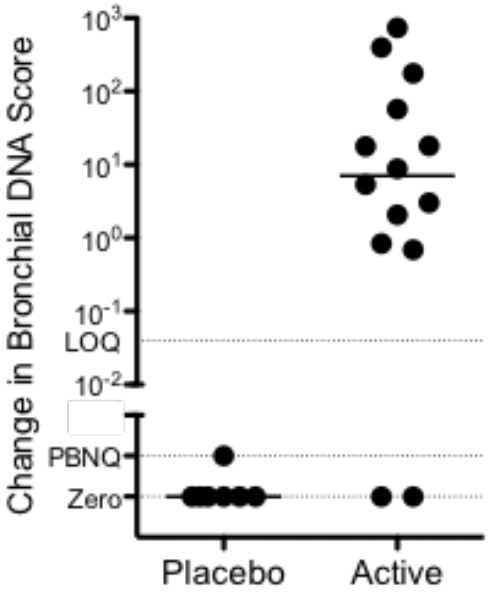


Figure 5.8. Graph summarising measurements of DNA from bronchial brushing from each bronchoscopy patient and the median group value (placebo: n=6; active: n=14). LOQ – limit of quantification; PBNQ – positive but not quantifiable (PBNQ); ND – not determined.

5.5.1 Relationship between change in *CFTR* cDNA and LAPD chloride efflux

To explore whether the patients demonstrating increases in *CFTR* transgene cDNA in bronchial epithelium from the active group had electrophysiological evidence of increased CFTR function, as measured by an increase in LAPD total chloride efflux, the two parameters were correlated. This correlation was made in the active patients only, in those completing 9 or more doses, with follow-up measurements taken within the accepted window, and having LAPD total chloride measurements at follow-up. There was no statistically significant correlation identified between vectors-specific DNA measured from the bronchial epithelium with the total tCl^- measured by LAPD at follow-up, in response to active treatment (r=-0.002, p=0.98; n=9, Spearman rank) (Figure 5.9).

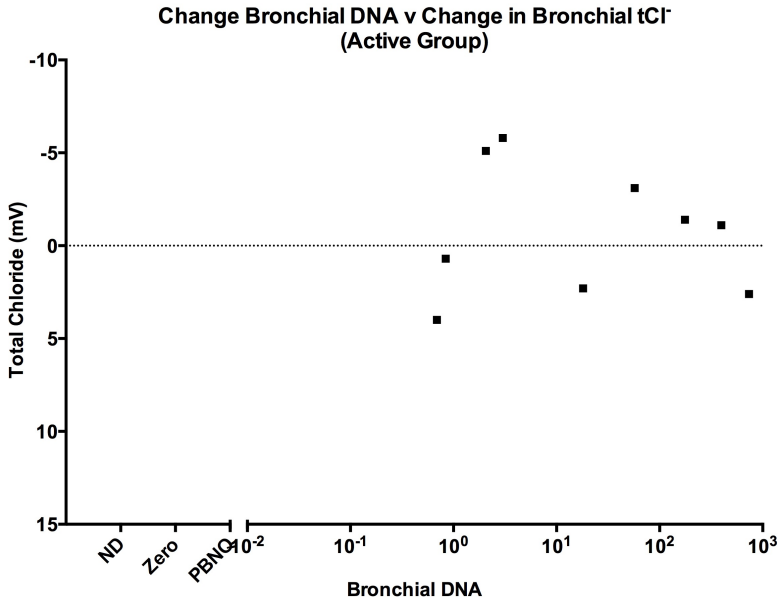


Figure 5.9. Graph plotting the correlation between bronchial cDNA and LAPD measured total chloride in response to active treatment (n=9).

5.5.1.1 Relationship between the lower airway cDNA and lung function

Further correlations were made between the total measured cDNA from the active group at follow-up with the changes in lung function ($ppFEV_1$ and LCI), with no statistically significant correlation being identified for either parameter: $ppFEV_1$: r=0.46, p=0.16; LCI = 0.356, p=0.30 (n=11) (Spearman rank).

5.6 Relationship between changes in lower airway electrophysiology and change in lung function measurements

LAPD is a direct assessment of CFTR function in the lower airway, and is felt likely by the author that it is more closely related to measurement of airway physiology (FEV_1 and LCI) than NPD, since all measurements are made within the same organ.

To understand whether any changes in lung function (specifically those of airflow obstruction, i.e. FEV_1 and LCI), in response to treatment with gene therapy, were associated with changes in LAPD as a measure of improvements in CFTR function, the following correlations have been made at baseline and in response to treatment.

5.6.1 Relationship between LAPD and lung function at baseline

Correlation analysis was performed between LAPD parameters (basal and total chloride response) and FEV_1 and LCI, at baseline as below.

5.6.1.1 Mean basal LAPD measurement with FEV_1 at baseline

A correlation was sought between baseline (i) mean proximal, and (ii) mean distal basal LAPD with mean $ppFEV_1$. At baseline, 22 patients had baseline LAPD measurements available for analysis. No correlation was found for mean basal measurements taken from the proximal or distal airway and baseline FEV_1 (Figure 5.10).

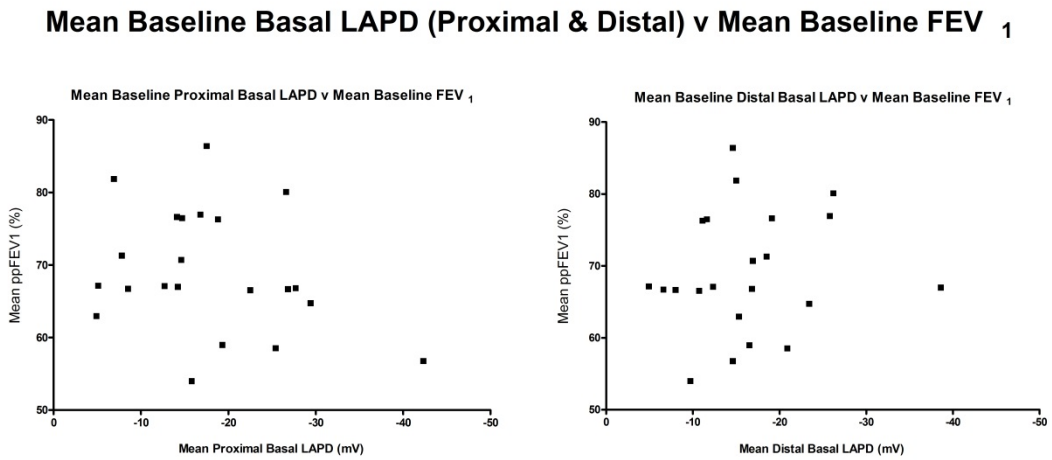


Figure 5.10. Graph of correlation between mean baseline basal LAPD and mean baseline FEV_1 (n=22), at (i) LEFT: proximal airway (r=0.28; p=0.21), (ii) RIGHT: distal airway (r=-0.13; p=0.56) (Pearson correlation).

5.6.1.2 'Most negative' basal LAPD measurement with FEV_1 at baseline

A further analysis using the most negative basal PD (taken from either site) at baseline was made with baseline FEV_1; once again no correlation was identified between the two parameters (r=0.22, p=0.33, Pearson correlation) (Figure 5.11).

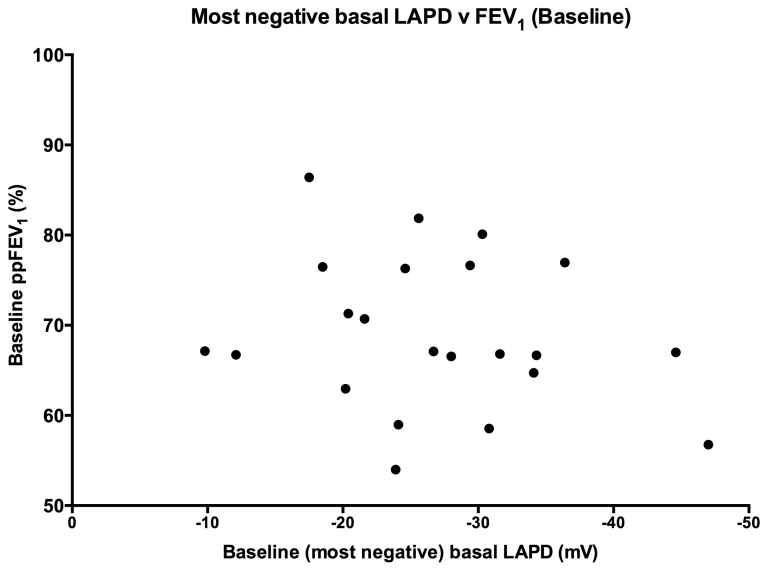


Figure 5.11. Graph displaying the most negative basal LAPD measurement at any site with the corresponding FEV_1 at baseline (n=22).

5.6.1.3 LAPD total chloride efflux with FEV_1 at baseline

At baseline, no correlation was found between mean baseline LAPD tCl^- and mean baseline $ppFEV_1$ (r=0.19; p=0.40, Pearson correlation) (Figure 5.12).

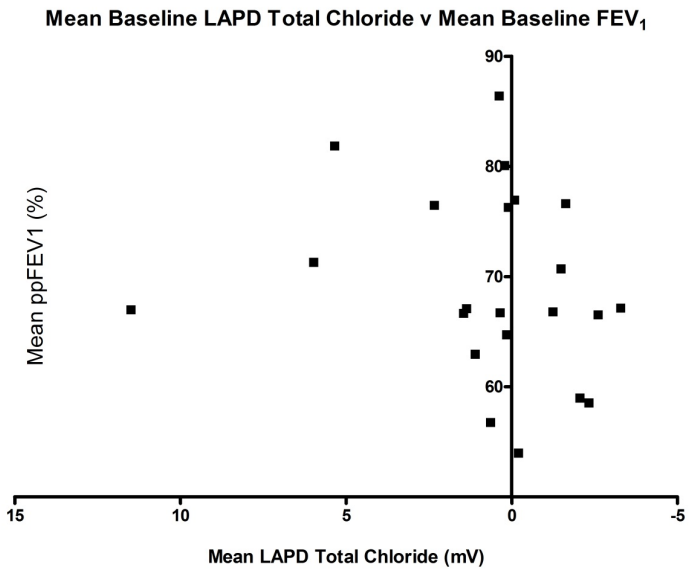


Figure 5.12. Graph displaying the relationship between mean baseline LAPD tCl^- secretion and mean baseline FEV_1 (n=22) (r=0.19; p=0.40).

5.6.1.4 Mean basal LAPD measurement with LCI at baseline

A statistical relationship had been demonstrated between basal NPD and LCI. To understand whether a relationship existed between the LAPD and LCI, a correlation was tested between the (i) mean proximal and (ii) distal basal LAPD and mean LCI at baseline.

At baseline, data from 21 subjects were available for analysis, owing to a failed LCI measurement in one subject (LON-4058). No correlation was identified either from the proximal or distal airway mean basal LAPD against the baseline mean LCI measurement (Figure 5.13).

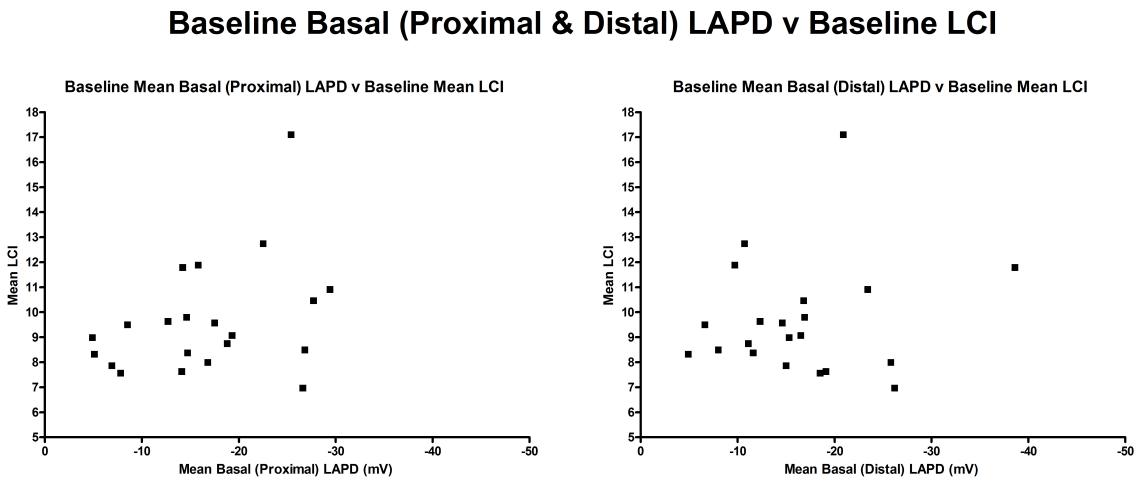


Figure 5.13. Graph at baseline of mean basal LAPD measurements against mean LCI, taken in the (i) LEFT: proximal airway (n=21) (r=-0.35), and (ii) RIGHT: distal airway (n=21) (r=0.06; p=0.80) (Spearman correlation).

5.6.1.5 Most negative basal LAPD measurement with LCI at baseline

As has been done previously, a further correlation was sought between the most negative basal measurement (from either site), with no statistically significant correlation was identified (r=-0.18, p=0.44 (n=21); Spearman correlation) (Figure 5.14).

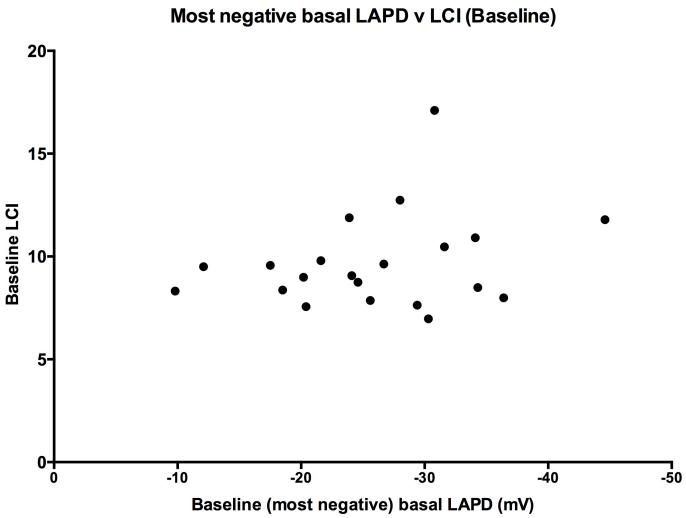


Figure 5.14. Graph showing most negative basal LAPD measurement at baseline and the baseline LCI (r=-0.18, p=0.44; n=21).

5.6.1.6 LAPD total chloride efflux measurement with LCI at baseline

At baseline (n=21), there was no correlation observed between mean LAPD tCl^- and mean LCI (r=-0.28; p=0.22, Spearman correlation) (Figure 5.15).

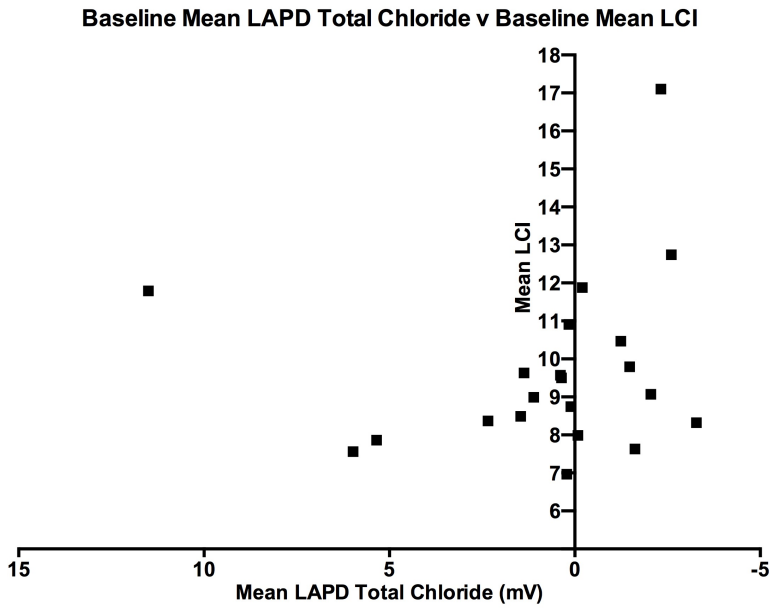


Figure 5.15. Graph at baseline of mean LAPD tCl^- measurements against mean LCI (n=21) (r=-0.28; p=22).

5.6.2 Relationship between LAPD and lung function in response to treatment with gene therapy

The same LAPD parameters were tested with FEV_1 and LCI at follow-up. The next section will look anecdotally for correlations owing to the small number of patients presented.

5.6.2.1 Changes in basal LAPD and FEV_1

There was no correlation between the mean change in both proximal and distal basal LAPDs with the mean change in $ppFEV_1$ in response to treatment with gene therapy (n=10) (Figure 5.16). No correlation was attempted with the most

negative LAPD basal measurement, as no relationship with this parameter had been previously identified.

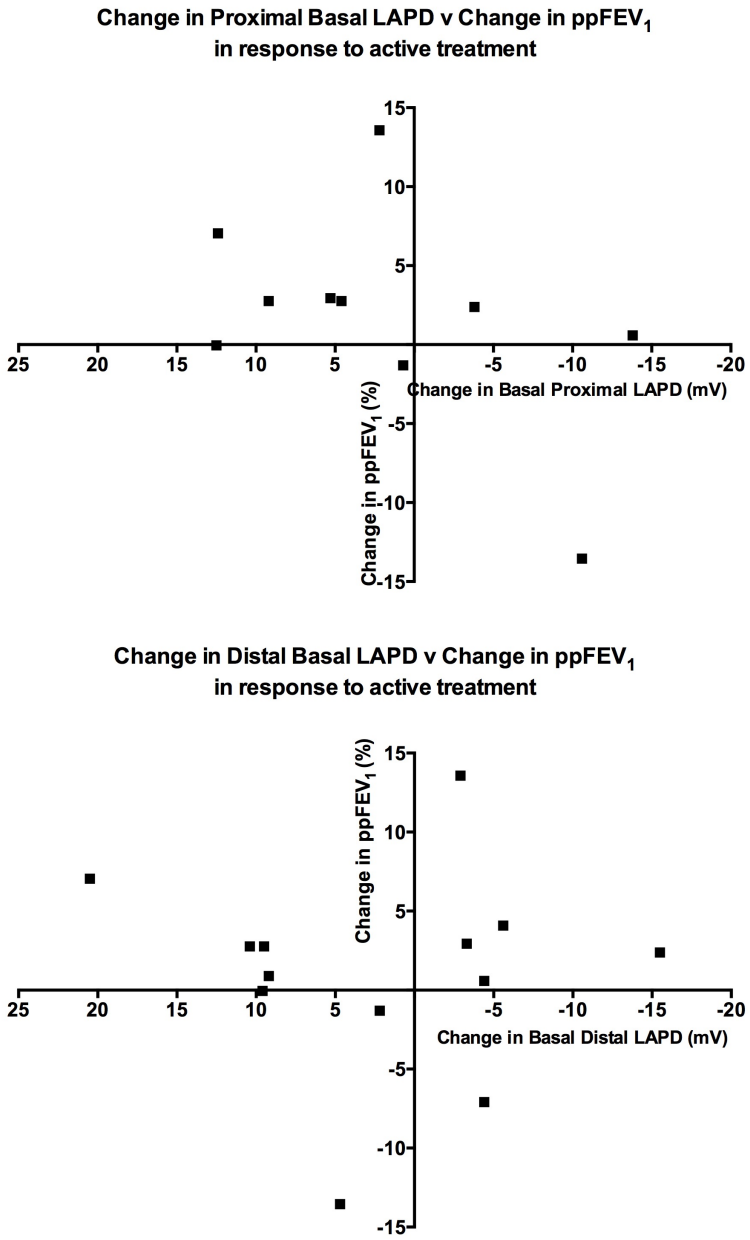


Figure 5.16. Graphs of the change in basal LAPD against the change in FEV_1 from baseline in response to gene therapy (n=10): (i) TOP: proximal basal LAPD (r=0.48, p=0.16); (ii) BOTTOM: distal basal LAPD (r=-0.01, p=0.97) (Pearson correlation).

5.6.2.2 Changes in mean LAPD total chloride and mean FEV_1

The results from 9 actively treated patients were available for analysis, owing to absence of baseline data, withdrawal from the study, delayed end of trial bronchoscopy or inability to perform follow-up spirometry. From this subgroup, the baseline $ppFEV_1$ was (i) 50-70% in 3/9 patients; (ii) 70-90% in 6/9 patients. Only 2/9 patients (one from each FEV_1 subgroup) demonstrated an improvement in $ppFEV_1$ of >5%, with only one of these demonstrating a hyperpolarisation in tCl^- response. No correlation was found between mean change in LAPD tCl^- and mean change in $ppFEV_1$ (Figure 5.17).

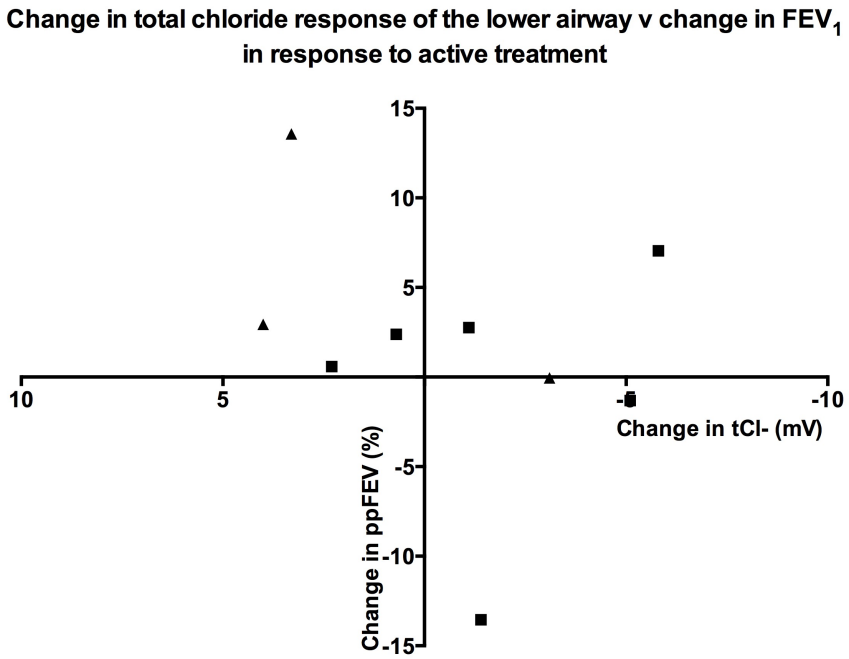


Figure 5.17. Graph displaying the change in total LAPD chloride from baseline and change in $ppFEV_1$ following treatment following active treatment (r=0.25, p=0.52; n=9) (Pearson correlation). (Patients with a baseline $ppFEV_1$: 50-70% are represented by a triangular data point; 70-90% by a square data point.)

5.6.2.3 Change in mean basal LAPD with change in mean LCI

No trend or clinically meaningful change between the two measurements was noted and neither was any significant correlation detected between LCI and basal LAPD in the actively treated group at either proximal airway (r=0.07; p=0.84) or distal airway (r=0.19; p=0.60) (Pearson correlation) (Figure 5.18).

No further analysis of the most negative basal measurement in the lower airway was made, as previous analyses had not detected this as a useful measure.

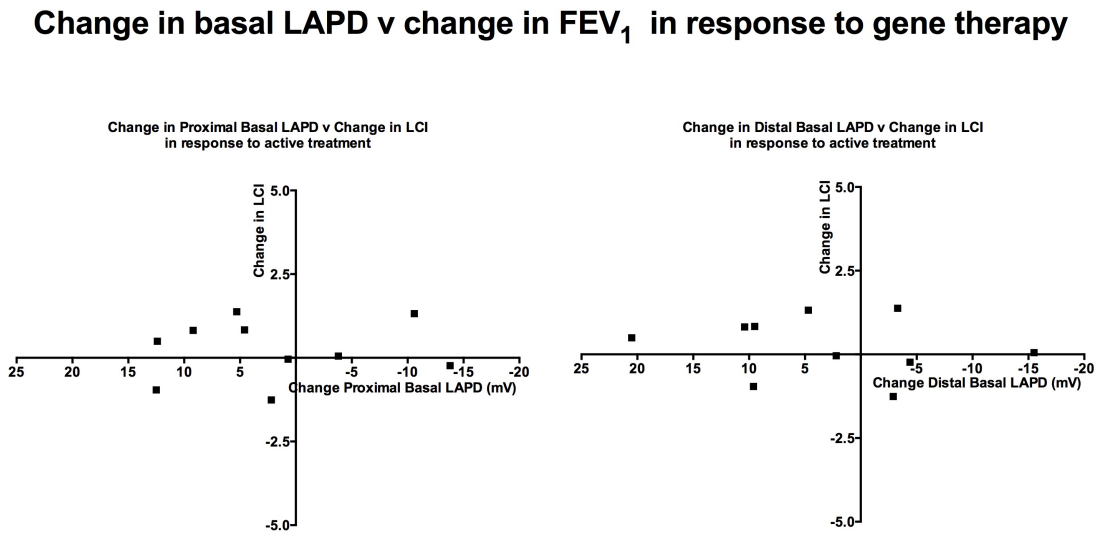


Figure 5.18. Graphs showing the change in mean basal LAPD against change in mean LCI in response to active treatment (n=10) at (i) LEFT: proximal airway; (ii) RIGHT: distal airway.

5.6.2.3 Change in mean LAPD total chloride with change in mean LCI

No statistically significant correlation was measured between the mean changes in these two parameters in response to gene therapy (r=0.02; p=0.93, Pearson correlation) (Figure 5.19). An improvement in CFTR mediated tCl^- secretion was matched by a small improvement in LCI in three cases; equally, subjects with improvements in tCl^- did not necessarily demonstrate improvements in LCI.

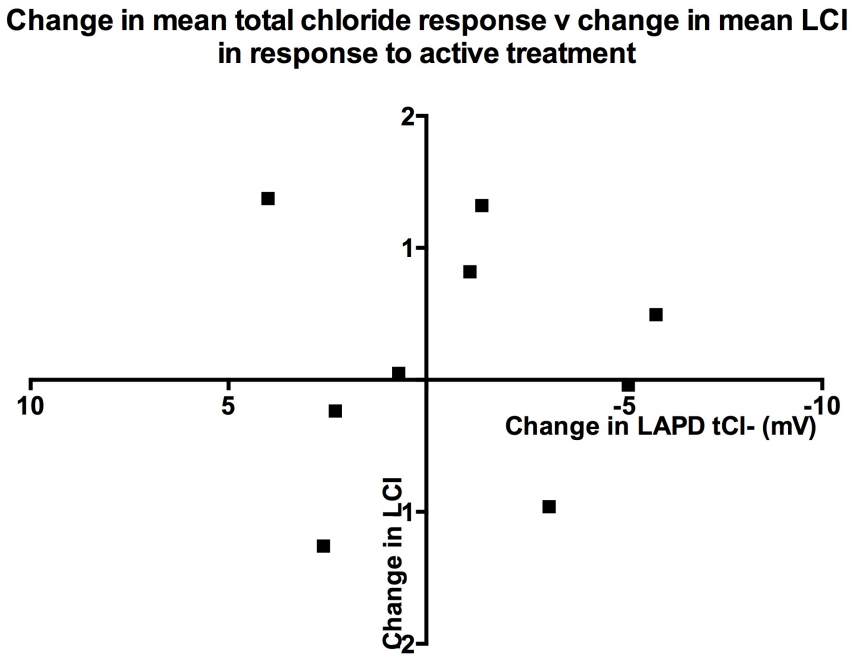


Figure 5.19. Graph displaying the change in LAPD tCl^- secretion against the change in LCI active treatment (r=0.02; p=0.93,n=9).

5.7 Relationship Between the Upper and Lower Airway PD

5.7.1 Patient Numbers

12 patients were enrolled into both nasal and bronchoscopic subgroups, however 2 subjects subsequently exited the study prior to randomisation and dosing; by performing 'extra' NPDs in the bronchoscopy-only subgroup, an additional 9 NPDs were performed. For ease of explanation, Table 5.6 clarifies the breakdown of the subjects included in this analysis. As this study is specific to this thesis, in place of the per protocol population being used, all patients undergoing bronchoscopy and NPD are included, including those withdrawing from the study pre-randomisation.

Both Subgroups			Bronchoscopy Subgroup Only		
Placebo	Active	Withdrawn (Never dosed)	Placebo	Active	
				NPD Pre-Dosing	NPD Post-Dosing
3	Baseline = 8 Follow-up= 5*	2	3	3	3

Table 5.6. Summary of population (n=22) having NPD and LAPD measured, by patient subgroup(s), treatment arm, and whether the NPD was performed pre- or post-dosing (*2 no/delayed follow-up bronchoscopy, 1 excluded from nasal subgroup).

From the 22 subjects having both nasal and lower airway PD measurements taken, 19 subjects had matched measurements taken from epithelium that had never been exposed to gene therapy (i.e. pre-dosing, or post-dosing and in the placebo group) – these 19 patients have been termed the 'untreated' population and have been used to allow baseline epithelial correlations. This group includes one subject (LON-4021) later withdrawn from the nasal subgroup owing to an excessive tCl^- secretion.

To test correlations betweens responses of the nasal and lower airway to treatment with gene therapy, only patients from the active treatment group who completed their follow-up measurements made within the protocol window were analysed. At follow-up, 5 subjects had received nasal and nebulised gene therapy and had pre-and post- nasal and lower airway measurements taken; any results from this very small group must be interpreted with caution.

5.7.2 Nasal and lower airway epithelial PD in the untreated airway

The following section will report measurements of the upper and lower airway PD, for basal and chloride response, in an 'untreated' population, i.e. having not been exposed to gene therapy.

5.7.2.1 Mean basal PD in the upper and lower airway

Correlations of basal PD were made between mean nasal and mean (i) proximal and (ii) distal lower airway PD (n=19). A moderate correlation, which did not quite achieve statistical significance, (r= 0.44, p=0.06 Spearman rank correlation) was observed between the nose and proximal lower airway (Figure 5.20), whereas there was no correlation between the nose and distal lower airway (r=-0.16; p=0.52, Pearson correlation) (Figure 5.21).

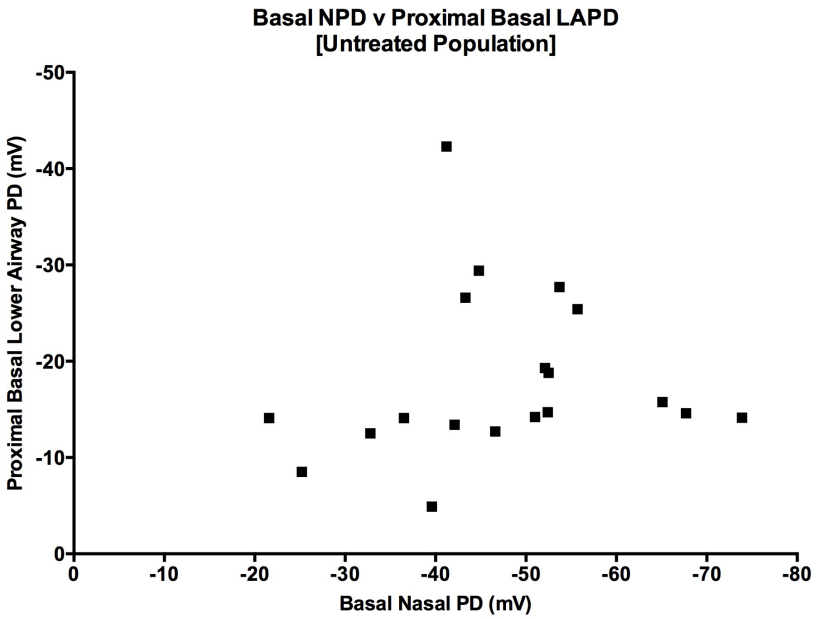


Figure 5.20. Graph from the untreated population (n=19) of basal NPD with proximal basal LAPD (r=0.44; p=0.06).

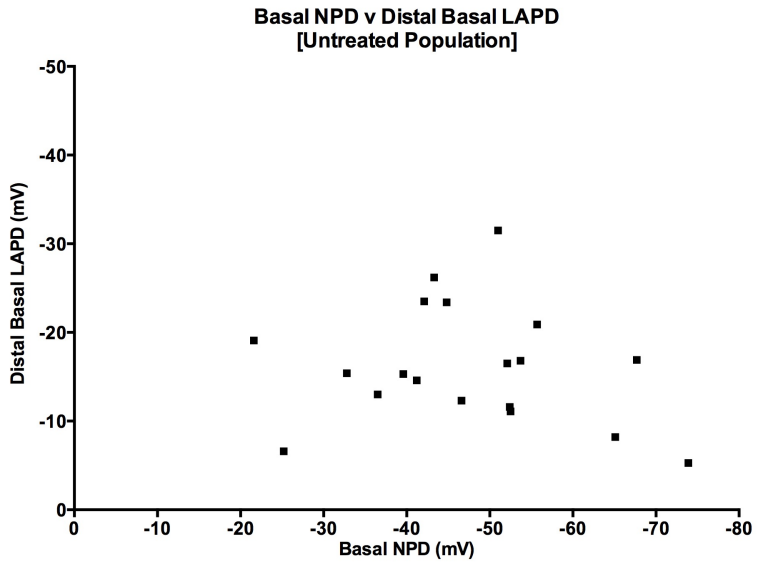


Figure 5.21. Graphs from the untreated population (n=19) of basal NPD with distal basal LAPD (r=-0.14; p=0.58).

5.7.2.2 Most negative basal PD in the upper and lower airway

In keeping with previous analyses, the most negative PD measured in the nose and in the lower airway were paired and tested for a correlation. No statistical correlation was found between the most negative basal PD from the upper and lower airway (r=0.05, p=0.84, Pearson correlation) (Figure 5.22).

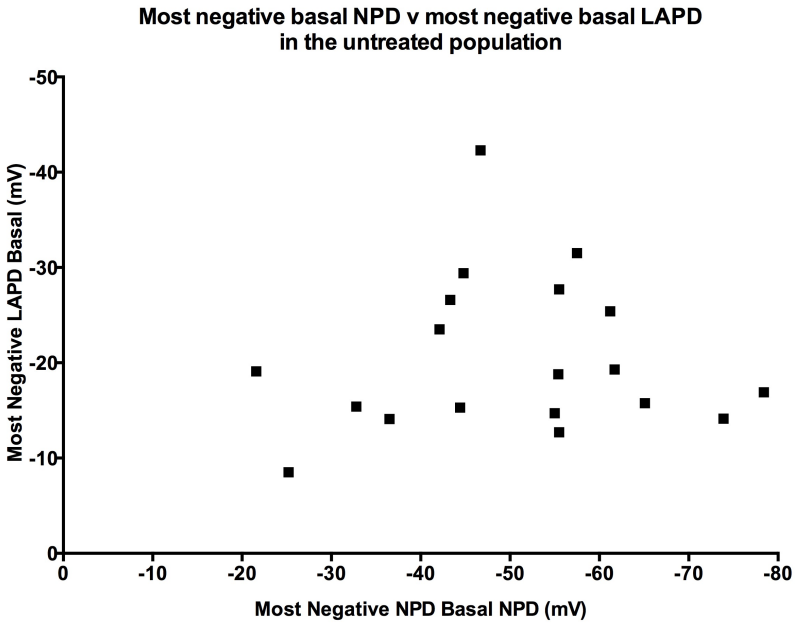


Figure 5.22. Graph showing the most negative basal PD from the nasal epithelium with those of the lower airway epithelium (n=19). (r=0.05, p=0.83)

5.7.3 Total chloride efflux in upper and lower airway

As expected, negligible tCl^- efflux was seen in both the upper (nasal) and lower airway at baseline. A few patients discussed in previous chapters have demonstrated residual CFTR-mediated chloride secretion by measuring a depolarisation (less negative) greater a -5 mV in their NPD and leading to withdrawal from the nasal subgroup – one of these subjects (LON-4021) entered the bronchoscopy subgroup.

A correlation was sought between tCl^- efflux measured at each site (n=19), and a moderate and statistically significant correlation was identified (r=0.49, 95% CI 0.026-0.776; p=0.04, Spearman rank correlation) (Figure 5.23).

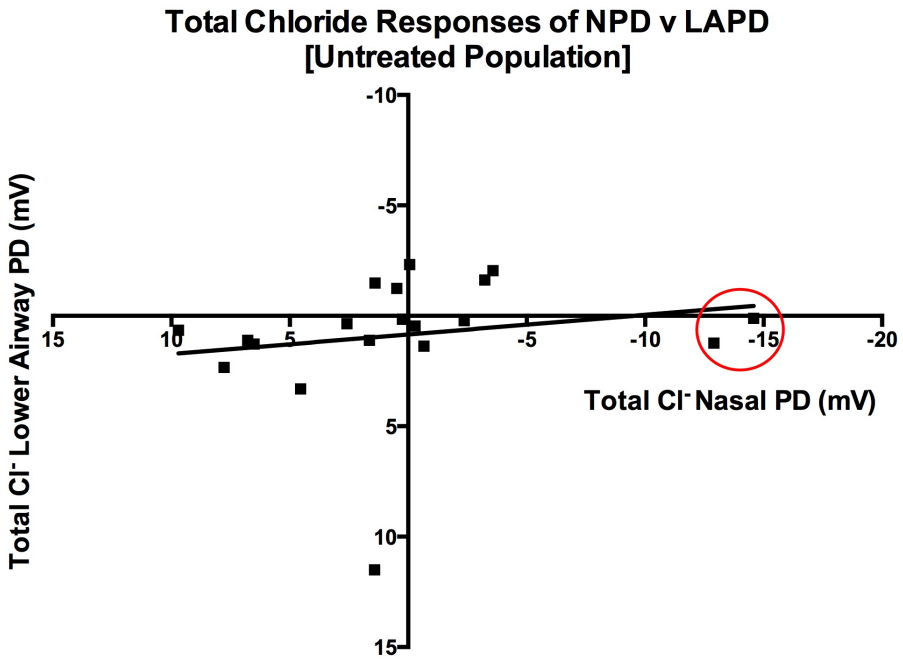


Figure 5.23. Graph of the relationship between total chloride efflux measured by NPD and LAPD in an untreated population (n=19) (r=0.49; p=0.04). Red circle highlighting two outliers.

5.7.3.1 Residual chloride secretion in the nose but not lower airway

Two outliers are identified (circled red in Figure 5.23), both demonstrating a large, non-CF (greater than -5 mV) amount of tCl^- efflux as measured by NPD,

with neither demonstrating any chloride secretion from the lower airway. Both subjects (LON-4021 and LON-4070) were diagnosed in adult life, have mild airways disease (ppFEV$_1$ 85 and 86%) with infrequent exacerbations, are pancreatic sufficient and carry the *CFTR* mutations *Phe508del*/3849+10kbC>T and *Phe508del*/*D1152H* respectively. If these outliers are removed from the data set, a more significant correlation is measured (n=17, r=0.59, 95% CI 0.143-0.840; p=0.01, Spearman rank correlation).

5.7.4 Nasal and lower airway epithelial PD responses to gene therapy

As has previously been reported, a proportion of patients demonstrated changes in tCl^- response to gene therapy change. It was decided that together the responses of the upper and lower airway epithelium to active treatment would be assessed, however the total number of patients completing the study in both subgroups was too small (n=5) to allow for statistical analyses to be made, and only anecdotal reporting of data can be provided.

A comparison between the changes in basal PD at either site failed to demonstrate a trend. The changes in tCl^- response between the nasal and lower airway in response to gene therapy is shown in Figure 5.24, which possibly suggests a linear trend, however as the results from only 5 patients contributed to this final analysis no conclusions can be made.

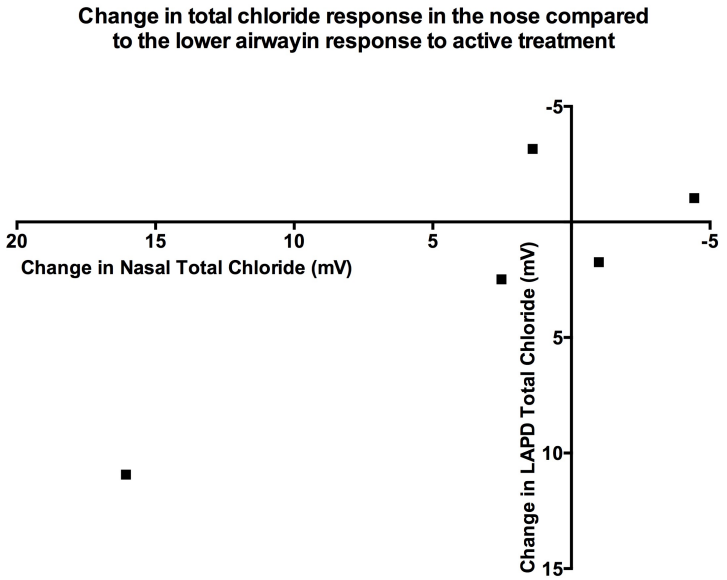


Figure 5.24. Change in total chloride secretion of the airway epithelium measured in the nose versus the lower airway in response in response to gene therapy (n=5).

5.8 Discussion

This chapter has reviewed the electrophysiology of the lower airway at baseline and in response to treatment with gene therapy, while examining the relationship of these parameters with changes in lung function and quantification of gene transfer to the epithelium. It has also made comparisons between PD measurements between the nose and the lower airway.

The chapter reported no statistical changes in basal LAPD in response to gene therapy, but did demonstrate a statistically significant treatment difference in total chloride response following active treatment. No statistically significant relationships were identified between LAPD parameters and measurements of lung function at baseline, or from the small number of patients (n=2) demonstrating spirometric improvements to active treatment. Quantifiable vector-specific DNA was measured in 85% of patients following active treatment,

compared with no patients in the placebo group; no mRNA was measured in any patient from either treatment group. The results will now be discussed.

5.8.1 Response of the LAPD to gene therapy

It would be expected that LAPD basal measurements would move in the direction of a non-CF individual, becoming less negative when sodium transport is normalised. However, as discussed it is unlikely that gene transfer is sufficiently efficient to restore ENaC function. It is therefore unsurprising that no change in basal LAPD was recorded in response to treatment.

Unlike in NPD, there is no 'threshold' for differentiating normal *v* CF by the LAPD total chloride response; similarly there is no definition for defining a treatment response. In comparison with the Consortium's Pilot Study, small treatment responses were seen (mean change -2.4 mV) (n=10), though it is important to remember these measurements were taken at 6 and 14 days following dosing with 20 ml of gene therapy [176].

The Multidose Trial demonstrated a statistically significant difference following gene therapy, with a treatment effect of -4.4 mV after 12 months of treatment. It is apparent that this was a combination of a small increase in tCl^- efflux (less negative PD) from baseline seen in the active group, and a small depolarisation in the placebo group. However, in such a small group, variations in even one or two recordings will markedly alter the interpretation. For example, the depolarisation in the placebo group is largely driven by one individual's large depolarisation. When looking at individual rather than group responses, 30% of patients treated with gene therapy had a total chloride response hyperpolarisation of -5.0 mV, suggesting some lower airway CFTR function in these subjects.

As noted above, the small numbers, as well as the heterogeneity in age and disease severity of the subgroup, are major confounders for measuring statistical

changes. As LAPD measures a small area of epithelium compared with the actual area dosed, it is possible that the tCl^- changes were 'missed' by taking measurements from an area of lung that had a lesser degree of transfection.

5.8.2 Comparison of LAPD with molecular *CFTR* results

All but 2 patients in the bronchoscopy active subgroup (85.7%) had measured increases in *CFTR* DNA at follow-up, compared with no patients in the placebo arm; it is not surprising that one of the active patients who had brushings taken at 111 days following their final dose had a negative molecular result which is outside of the life-span expected for vector-specific DNA.

Again no pGM169-derived mRNA was measured in any patient at follow-up, which likely reflects the low sensitivity of this assay and has been reported in previous *in vivo* studies [210].

5.8.3 The relationship between LAPD and measurement of lung function

No correlations were found at baseline between any LAPD parameter and either FEV_1 or LCI. It is surprising nonetheless that relationships had been identified between NPD and lung function (chapter 4), and yet when measurements were made from the same organ no association was found. The major confounders for these results are the heterogeneity in severity of lung disease (wide ranges of FEV_1 were seen at baseline (54 – 86%)), and the small numbers of patients studied, making any true correlations very hard to verify statistically. As expected, a relationship between chloride responses and lung function was not demonstrated, owing to negligible chloride secretion in the CF airway with diverse FEV_1 and LCI measurements. It is possible that some of these findings reflect LAPD being made from very specific areas whereas both lung function measurements represent the entire airways.

Furthermore, no relationships were identified in response to treatment; of most importance improvements in tCl^- response did not translate into clinical benefit, measured by improvements in FEV_1. As only few patients demonstrated improvements in lung function, it difficult to confirm statistical relationships between improvements in these two parameters. As was reported in chapter 2, only patients with more severe lung disease ($ppFEV_1$ 50-70%) demonstrated improvements in lung function in response to active treatment – the numbers in this subgroup were already too small to study this parameter further. As the overall number of patients treated with gene therapy is small (n=9), the group is underpowered for statistical analyses and conclusions between electrophysiology of the lower airway and clinical benefit should remain undetermined.

Alternatively, it is possible that gene therapy and *de novo* CFTR function per se are not responsible for the improvements in lung function. Alternative mechanisms cannot be excluded – it was observed that a single dose of pGM169/GL67A reduced airway inflammatory markers and bacterial adherence to the airway epithelium [144], and therefore an interaction between the gene-liposome complex and the innate immune response should be excluded in future studies.

5.8.4 Comparison of basal LAPD between adults and children and at proximal and distal airway sites

It had been demonstrated in children (with a mean (SD) age of 9.1 (1.8) years, n=15) and adults with CF that basal PD progressively falls as serial LAPD measurements are made more distally throughout the lung (Figure 3.11) [208]; no comparison between adults and children has been previously reported. The same study confirmed that healthy controls had a lower basal PD, reflecting normal sodium transport in the lower airway.

Within the MDT bronchoscopy cohort, two methods were used to contrast results with this previous finding – firstly using all patients' basal results, and secondly using only the most negative measurement from each patient. Both methods provided similar outcomes, however statistically significant differences were observed when using all basal measurements: a more negative basal PD was measured at the proximal compared to the distal airway in all patients, and in patients over 18 yrs (adults), however this differentiation was lost in measurements from children. No significant differences were observed in the basal LAPD measurements at either site between children and adults.

The author suggests several possible reasons why the differentiation of basal LAPD at proximal and distal sites in children was less discriminatory than the results from Davies *et al*. Firstly, the numbers of children having basal PD measurements in the MDT was small (n=6), and almost a third of those previously reported; this number is likely too small to demonstrate a true difference between the sites owing to high inter-subject variability. Secondly, the children studied in the MDT were slightly older than those previously reported (mean (SD) age 9.8 (1.8), with the youngest child being 1 yr old) with a mean (SD) age of 12.7 (0.8) years, and it is possible that less differentiation occurs in adolescents; it is not possible to comment on the severity of lung disease in these children, as this value was not reported in the original study. Finally the variability of LAPD measurements is important to consider, particularly relating to any local airway inflammation that may be present, altering ion transport and attenuating basal PD and impacting on the measured results.

5.8.5 Relationship between the PD of the upper and lower airway CF airway epithelium

Correlations from an 'untreated' population of CF patients were made between the basal PD of the nasal mucosa and those of the lower airway at both sites, using both mean and most negative values. No relationship was identified at either site or by either stratification. This would suggest that basal PD

measurements from the lower airway cannot be predicted from those measured in the nose.

As CF leads to absent or negligible transepithelial chloride transport, examining a relationship for this parameter in two sites is difficult. However, despite the small patient numbers, and the low levels of tCl^- responses, a statistically significant correlation was identified at baseline between the nose and the lower airway.

The results suggest that patients having small, residual movements of airway chloride do so in both airway sites. It is difficult to draw any conclusions about the changes in chloride transport between the upper and lower airway following gene therapy, as whilst a trend was suggested the patient numbers are too small (n=5).

The upper airway is a continuous extension of the (lower) respiratory tree, and susceptible to infection and inflammation in CF, so it could be expected that they would behave similarly. The small numbers of patients analysed significantly limits this study, and therefore conclusions about epithelial relationships cannot be drawn with confidence.

Of interest however, two patients demonstrate large nasal tCl^- responses (-12 and -15 mV) with both having no tCl^- movement measured in the lower airway. Both patients have mild CF disease with one *CFTR* allele bestowing a protein with residual function (*D1152H* – class IV mutation and 3849+10kbC>T – class V mutation [225]). It would be unexpected that patients would have different expression of CFTR in each organ, therefore it could be hypothesised that these patients have a different CFTR response to the chloride-free perfusate used – that difference being the absence of amiloride in solution used in the lower airway. It would be interesting in future work to measure these patients' NPD again using zero chloride solutions with and without amiloride. The next chapter will address the influence of amiloride on chloride transport. As conclusions are limited when considering two patients, the understanding of preserved chloride

secretion in the upper and lower airway, and in its role in survival, could be further advanced in future studies by taking measurements from CF patients with a NPD tCl^- response > -5 mV and measuring their equivalent response from the lower airway.

5.9 Conclusions

This chapter has reported evidence of *CFTR* DNA being transferred into the lower airway epithelium, and reported individual patients who have demonstrated electrophysiological improvements in CFTR function supporting *de novo CFTR* expression. An overall statistically significant difference in treatment effect was seen following 12-months of non-viral gene therapy, therefore author accepts the first part of the original hypothesis. From the small number of patients studied and the variation in treatment responses observed, limited conclusions can be drawn for relationships between gene transfer and clinical benefit, as measured by lung function, of *de novo* CFTR function and thus rejecting the second part of the hypothesis. It is possible that areas of lung have inhomogeneous 'uptake' of pGM169, and that these do not correspond with those assessed by bronchoscopy. It has already been suggested that increased airway mucus is a possible reason for limitation of gene uptake, and these areas were purposefully avoided during the bronchoscopy because of the recognised interference with LAPD measurements and operator visibility. Finally, it is possible that the chosen dose (5 ml) of pGM169/GL67A is sufficient to cumulatively produce small changes in airway function, yet a higher dose of DNA-liposome complex is needed to categorically demonstrate epithelial CFTR function in the lower airway, particularly given the variability seen in LAPD measurements.

I would suggest that future gene therapy trials should statistically power a bronchoscopy subgroup to assess electrophysiological changes, and that LAPD responses from patients with milder and more severe lung disease (FEV_1 70-90% *v* 50-70%) are studied. It would be suggested in future studies that LAPD

measurements are taken from different bronchial distributions of the lung within the same individual, to 'sample' a wider area of epithelium and reduce the possibility of measuring from an under-treated airway.

Chapter 6: Assessment of the nasal and lower airway PD protocols

6.1 Background to Assessing Nasal and Lower Airway PD Protocols

This chapter will present a study assessing the differences in protocols used in the measurement of nasal and lower airway PD. The author thought it important to understand whether differences between the protocols could lead to underestimates of electrophysiological measurements in the lower airway. There are two main differences: i) during the Cl^- secretion phases of the NPD protocol, perfusing solutions contain amiloride, whereas in the LAPD protocol they do not, and ii) in the NPD protocol, chloride secretion is measured over two periods of 5 min and summed, whereas only 5 min is used in the lower airway.

The hypotheses of the study were: (i) CFTR-chloride transport was time dependent, and that longer PD perfusions would produce greater tCl^- responses; (ii) that the presence of amiloride in the perfusate would alter the transmembrane electrochemical driving force (the effect of membrane potential and concentration gradient (luminal compared to intracellular) created by Na^+ and Cl^- ions) [226] [227], and that in the presence of ENaC blockade the epithelium would have an increased permeability to Cl^- (i.e. more chloride secretion).

The aim of this study was to compare nasal epithelial tCl^- efflux measured by NPD, perfusing 'standard' NPD solutions and LAPD solutions over the 'standard' or an extended time duration. To investigate these, the author designed and conducted a study comparing the protocols, making measurements in the noses of healthy volunteers; healthy controls were used as the measurement of CFTR-mediated chloride efflux was required, which is absent in CF patients.

6.2 Method to Compare the Nasal and Lower Airway PD Protocols

6.2.1 Healthy volunteer recruitment

Healthy (non-CF) volunteers (>18 yrs) were recruited; initially 8 subjects were planned, however extra subjects were needed, as it became obvious more data were needed to draw valid conclusions. All subjects were non-smokers and were self-reported to be free from recent (within 2 weeks) or current nasal symptoms (e.g. recent URTI, hayfever, sinusitis).

The National Research and Ethics Service (NRES) (London – Brent Committee, Ref. 13/LO/0391) approved the study and all volunteers provided full informed consent. Each subject was given a unique study identifier to maintain anonymity.

Each subject attended the CRF for at least 2 visits to have NPDs performed. There was no pre-determined interval between visits, although it was attempted to make the measurements as close together as was logistically possible whilst ensuring that volunteers were clinically stable; volunteers were not scheduled to have both NPD measurements performed on the same day.

6.2.2 Performing nasal PD measurements

The NPD equipment was set up and the subject prepared as described in chapter 3. The most negative site of baseline NPD was identified from both nostrils and this position used for all subsequent measurements.

6.2.3 The nasal and lower airway perfusion protocols

Two perfusion protocols were performed on each subject on different days; the order of each protocol being performed was varied randomly (but was mostly dictated by the availability of PD solutions and subject time). Any abnormal measurements, thought to result from technical errors or subject-related factors (e.g. a flat trace resulting from nasal inflammation from a recent but resolved URTI), could be repeated at the discretion of the author and agreement of the subject.

(i) **'NPD protocol'** – a standard nasal PD perfusion protocol. A standard NPD was performed (as fully described in chapter 3) with 4 solutions being perfused at 240 ml/hr: (i) Ringer's solution; (ii) Ringer's + amiloride (RA); (iii) zero chloride + amiloride (ZCA) for 5 min; (iv) zero chloride + amiloride + isoprenaline (ZCAI) for 5 min.

(ii) **'LAPD protocol'** – replicated the lower airway PD perfusion protocol. The two solutions used for the lower airway PD protocol (chapter 3) were administered but perfused via a nasal catheter and challenged the nasal epithelium. The solutions used were (i) Ringer's solution; (ii) zero chloride + isoprenaline (ZCI) solution – this solution, as previously mentioned, purposefully does not contain amiloride.

The LAPD protocol was initially performed at 240 ml/hr for 5 min, however once initial data were collected, the author chose to (1) slow the rate of perfusion to 100 ml/hr in a small number of subjects to match the perfusion rate administered onto the lower airway mucosa during bronchoscopy, to consider whether the rate of perfusion affected the PD response; and (2) continue perfusion for an additional 5 min, to assess whether the time of perfusion was a significant variable in chloride response.

6.2.4 Data recording and analysis

NPD data were saved electronically and analysed in Microsoft Excel, as previously described, measuring the total chloride secretion by each protocol.

6.3 Results

6.3.1 Subject recruitment and demographics

24 subjects were recruited for this project. Once an initial 8 subjects had been recruited, an interim analysis of the data failed to identify any trends owing to the wide variation of NPD measurements. In total, 6 subjects were excluded from the study, owing to blunted PD responses in 5 volunteers (it was felt that subjects under-reported the presence of any nasal symptoms, e.g. allergic rhinitis or incomplete recovery from an URTI) and one subject having an unexplained, positive basal value on their second PD.

The analysis was based on 18 subjects (8 male, 10 female) who completed the study, with a mean (SD) age of 32 (5.6) yrs.

6.3.2 Nasal PD measurements taken

The majority of NPDs were performed by MDW (KH performed 3 NPD measurements).

Of the 18 subjects included, 4 subjects had extra NPD measurements taken for either technical reasons (electrode drift, loss of electrical contact and slippage of nasal catheter) or for confirmation of unexpectedly low chloride responses (the greatest response was included in the analysis).

6.3.3 Measurement of duration and perfusion rate

From the 18 healthy volunteers, 13 had the lower airway solutions perfused at 240 ml/hr, and 5 had the LAPD protocol perfused at 100 ml/hr.

Following an initial 5 min LAPD perfusion with ZCI, 5 subjects volunteered to have an additional 5 min perfusion of ZCI solution to assess for any addition chloride efflux; all but 1 of the subjects had both of these perfused at 100 ml/hr.

6.3.4 Summary of all PD indices measured by each protocol

The following Table 6.1 summaries all of the mean basal and chloride responses (individual parameters and total secretion) from the two protocols; a further Table 6.2 shows the same PD responses but with subjects separated into the LAPD perfusion rate (240 ml/hr v 100 ml/hr)

Mean (SD) (mV)	**Nasal Protocol**	**Lower Airway Protocol**
Basal PD	-22.0 (5.6)	-20.0 (6.1)
dAMIL	12.0 (6.1)	
dZCA	-15.0 (11.7)	
dZCAI	-18.8 (8.0)	
tCl^- Response	-33.8 (16.0)	-21.6 (15.7)

Table 6.1. Summary of PD responses for standard nasal and lower airway protocol performed in the nose of healthy subjects (n=18).

	LAPD Perfusion at 240 ml/hr (n=13)		LAPD Perfusion at 100 ml/hr (n=5)	
Mean (SD) (mV)	**Nasal Protocol**	**Lower Airway Protocol**	**Nasal Protocol**	**Lower Airway Protocol**
Basal PD	-21.5 (4.4)	-20.4 (5.5)	-23.3 (8.4)	-18.9 (9.2)
dAMIL	10.7 (6.3)		13.9 (5.4)	
dZCA	-12.1 (9.2)		-19.8 (16.7)	
dZCAI	-19.1 (9.2)		-16.1 (2.6)	
tCl^- Response	-31.3 (15.5)	-16.8 (15.3)	-35.9 (18.7)	-29.4 (14.2)

Table 6.2. Summary of PD responses for standard nasal and lower airway protocol (at 2 perfusion rates) performed in the nose of healthy subjec§ts.

6.3.5 Basal PD Measurements from all subjects

The mean basal PD did not differ significantly in either protocol (p=0.46, n=18 (paired t-test)); there remained no significant difference when subjects were separated by LAPD perfusion rate (240 ml/hr: p=0.57 (n=13); 100 ml/hr: p=0.43 (n=5), paired t-test).

Figure 6.1 shows the basal PD measurements in all individual 18 subjects as measured by either protocol; a Bland-Altman plot (Figure 6.2) compares the basal PD measurement performed by each protocol on different days.

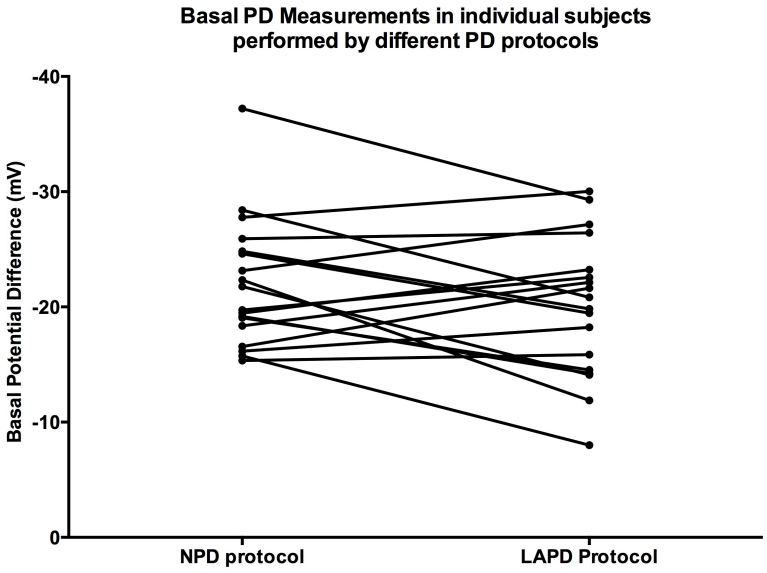


Figure 6.1. Basal PD measurements in the same individual performed on different days and by a different protocol (n=18).

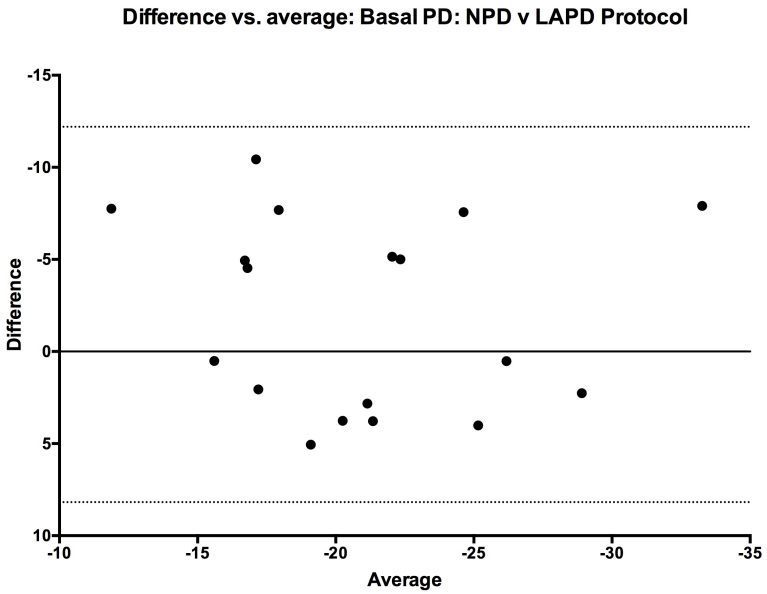


Figure 6.2. Bland-Altman plot of basal PD measurements made by nasal and lower airway PD protocols (n=18) (95% confidence intervals shown).

6.3.6 Variables in total chloride responses by each protocol

It was important to understand whether the perfusion rate or duration affected the total chloride response. Several variables of chloride efflux were measured in this study (compared with the NPD protocol perfused at 240 ml/hr for a total of 10 min):

(i) LAPD protocol perfused at 240 ml/hr for 5 min;
(ii) LAPD protocol perfused at 100 ml/hr for 5 min;
(iii) LAPD protocol perfused for 10 min (an additional 5 min).

The following sections will report these variables and the differences in chloride secretion with the two protocols.

6.3.6.1 Effect of perfusion rate on chloride efflux

There was no statistical difference between the tCl^- response between the NPD and LAPD protocols at either perfusion rate (p=0.21, paired t-test) – the paired tCl^- response measured at each rate are shown in Figure 6.3.

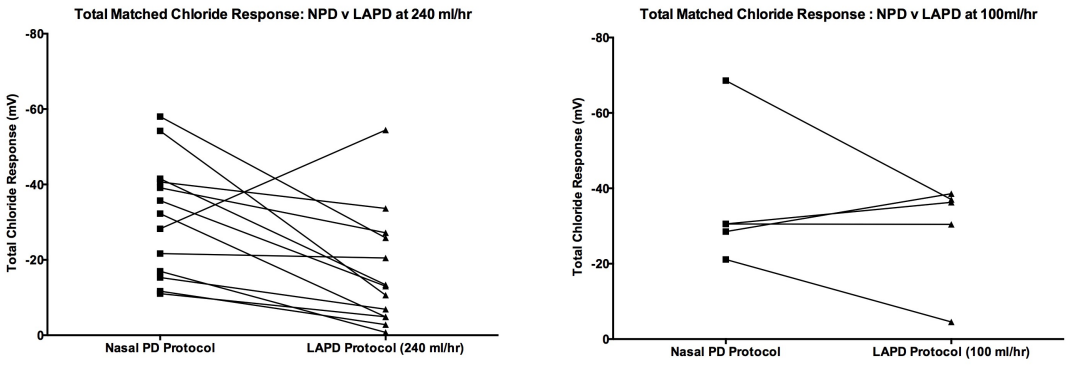


Figure 6.3. Total chloride responses measured in healthy volunteers by the NPD protocol, and LAPD protocol, perfused at (i) LEFT: 240 ml/hr (n=13), and (ii) RIGHT: 100 ml/hr (n=5). (p=0.21, paired t-test).

6.3.6.2 Relationship between chloride efflux obtained with the two protocols

Having reported that the rate of perfusion does not significantly alter the chloride efflux, all of the data from the 18 subjects were included in further analyses.

To compare the tCl^- responses measured by each protocol, the matched results for each subject have been plotted, the group data plotted and the mean calculated (Figure 6.4). It is apparent that whilst the range of tCl^- responses is similar between the two protocols, there is a statistically significant reduction by a mean of 12.2 mV in tCl^- with the lower airway protocol compared to the nasal protocol ($p<0.01$, paired t-test); 3/18 (16.7%) healthy volunteers had greater tCl^- secretion with the LAPD protocol than with the NPD protocol, with one subject almost doubling their tCl^- responses (-28.2 *v* -54.4 mV).

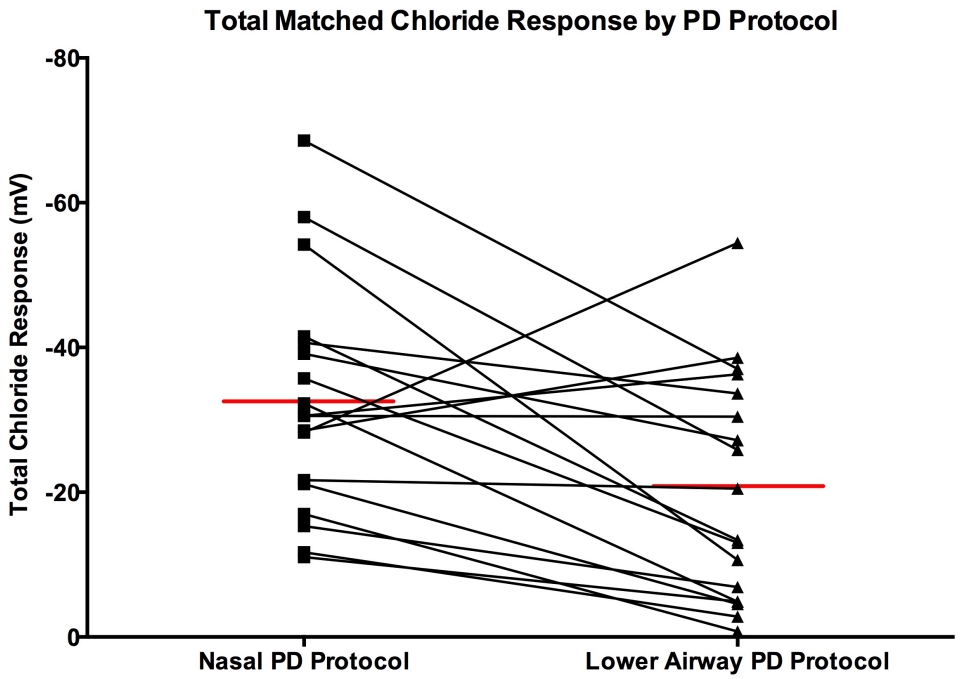


Figure 6.4. Matched individual total chloride measurements by each protocol (n=18), mean values for each are shown in red ($p<0.01$, paired t-test).

The relationship between the total chloride secretion measured by the LAPD protocol and NPD indices was assessed, with no significant correlation being measured (r=0.41, p=0.09, (Pearson correlation)) (Figure 6.5) – the graph indicates subjects having LAPD perfused at a slower rate. No correlations were detected between the tCl^- measured by the LAPD protocol and the NPD protocol (i) ZCA phase: r=0.30 (p=0.23), and (ii) ZCAI phase: r=0.39 (p=0.12).

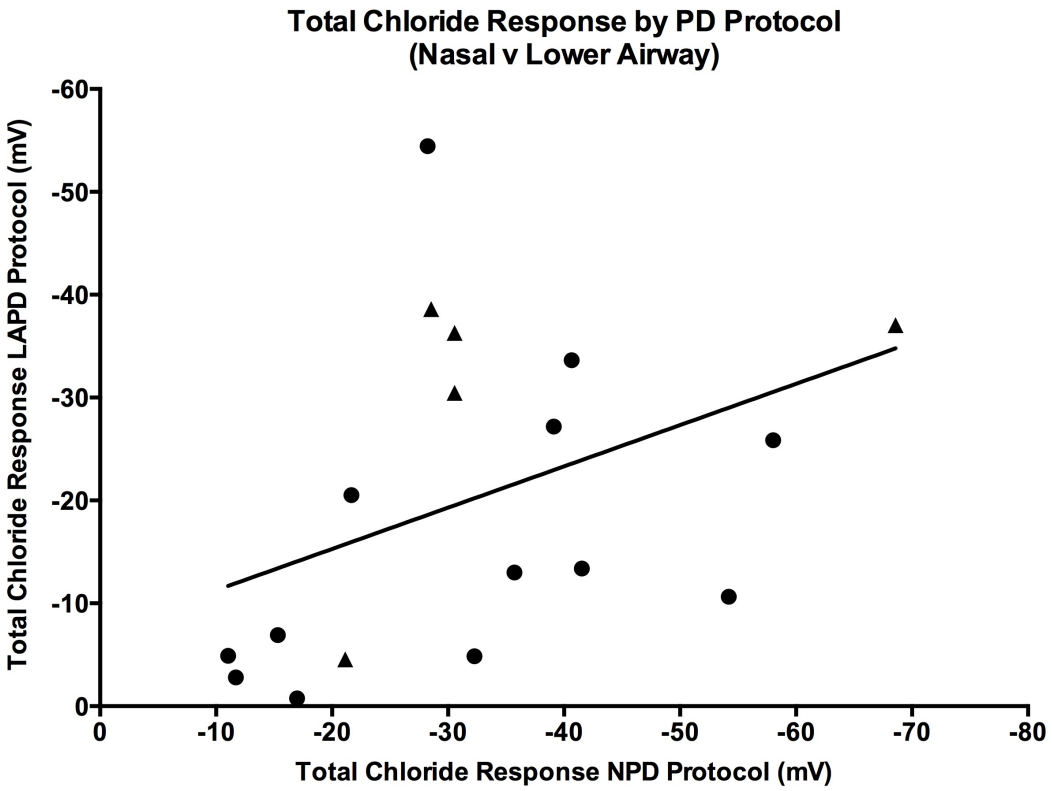


Figure 6.5. Scatter-plot & trend-line for the total chloride response per subject for the nasal protocol against the lower airway protocol performed in the nose of healthy subjects (r=0.41, p=0.09, n=18 (Pearson correlation coefficient)). Circular data points represent subjects having both perfusions as 240 ml/hr, triangular data points represent subjects having LAPD solutions perfused at 100 ml/hr.

6.3.6.3 Effect of duration of stimulation on chloride efflux with the lower airway protocol

To understand whether the LAPD protocol could underestimate tCl^- compared to the NPD protocol resulting from a reduced perfusion time, 5 subjects had an additional 5 min perfusion with ZCI and any additional chloride efflux was quantified. An additional tCl^- response to a further 5 min perfusion of ZCI was seen in all subjects, with a mean (SD) response of -7.1 (5.7) mV, and a range of -2.2 to -15.6 mV. The NPD trace from the subject demonstrating the greatest additional tCl^- response is shown in Figure 6.6 – note that the chloride response in this individual still has not reached a plateau after 10 min.

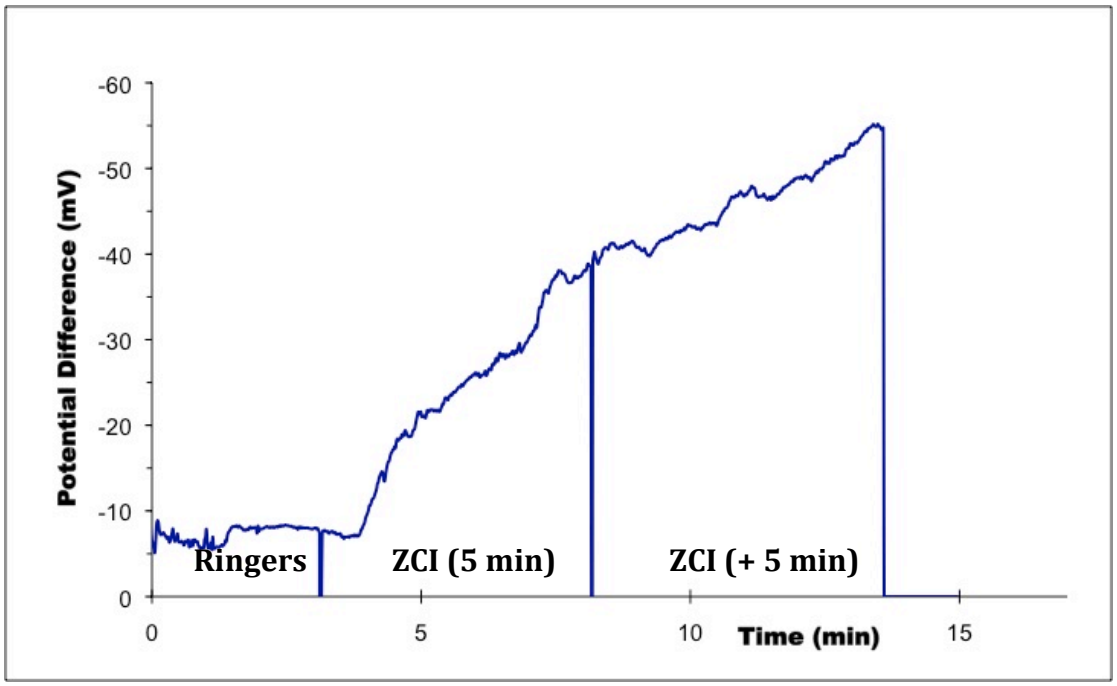


Figure 6.6. Nasal PD trace from a healthy volunteer (subject 3) with total chloride responses following perfusion with zero chloride+isoprenaline solution for 5 min and an additional 5 mins.

To contrast the total Cl^- responses measured in these 5 subjects, a bar chart (Figure 6.7) shows measurements by the standard NPD protocol and the LAPD protocol at 5 min and with an additional 5 min. The graph highlights variability in these subjects between the tCl^- response measured, with 2/5 subjects already having more tCl^- with a short LAPD protocol than nasal and 1/5 being the same,

and 2/5 subjects having a tCl^- response less than their NPD despite 10 mins of ZCI perfusion.

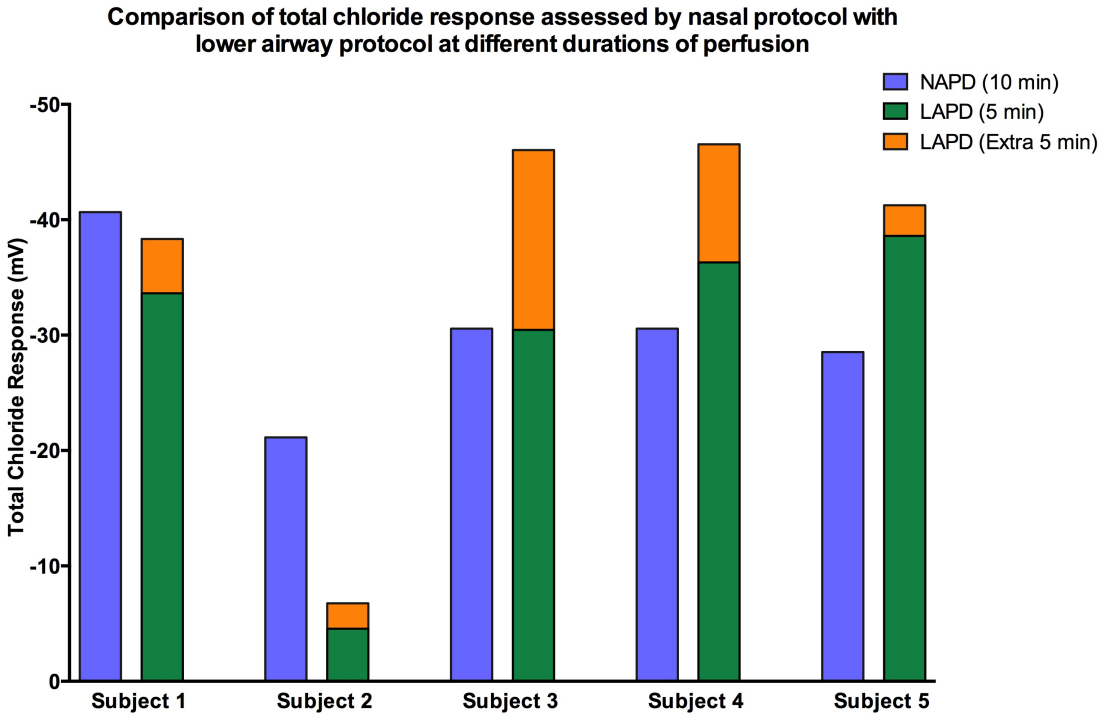


Figure 6.7. Comparison of total chloride responses assessed by the NPD protocol (after 10 min perfusion) with the LAPD protocol at 5 min and following an additional 5 min perfusion with ZCI (n=5).

6.3.6.4 Relationship between dAmil and total chloride response

To help understand whether the absence of amiloride in the perfusate directly influenced total chloride secretion, a correlation was tested between the dAMIL response (in the nasal PD protocol) with the difference in the tCl^- secretion between the two protocols (Figure 6.8), with no correlation being identified r=0.09, p=0.73, Pearson correlation).

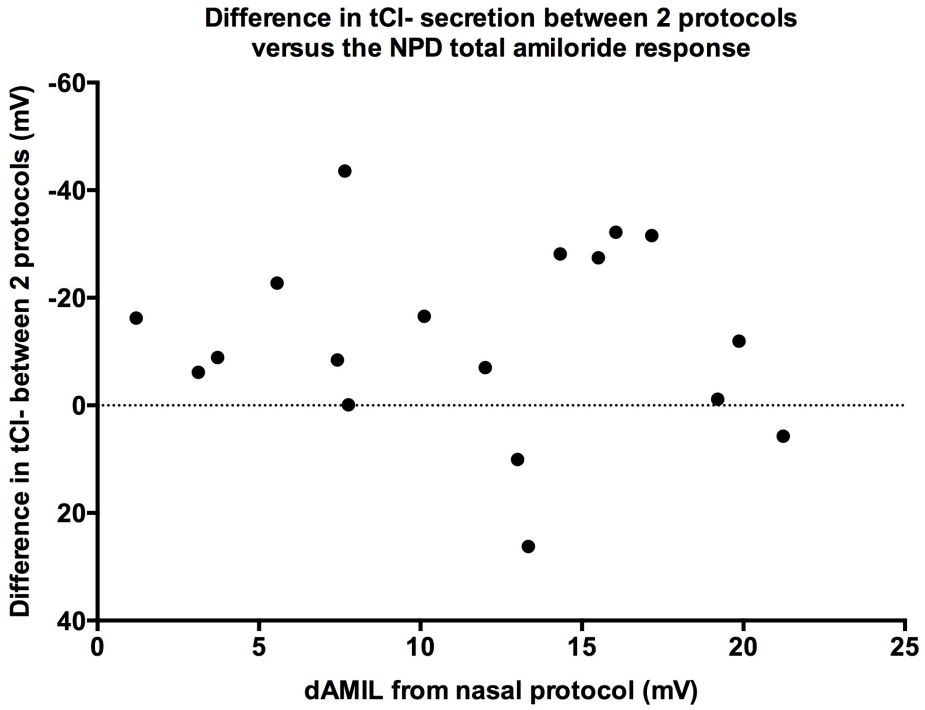


Figure 6.8. Plot of nasal dAMIL response against the difference in total chloride response between the 2 protocols (r=0.09, p=0.73).

6.4 Discussion

In this chapter, I have presented a study investigating the variables that exist between PD protocols in the nose and lower airway and demonstrating variations in chloride secretion between these two methods, which may account for the differences seen in epithelial PD response. This section will discuss these results, and the possible influence of this study on interpretation of the MDT data.

To assess whether tCl^- responses could be underestimated by the LAPD protocol, a comparison of the two techniques was performed in the nose of healthy volunteers (i.e. with functional CFTR). An unexpectedly high number of subjects was excluded from the study (6/24 (25%)) owing to flat PD responses, but on further questioning subjects had recent or undisclosed UTRIs or hayfever. The author relates this phenomenon to the generosity of the volunteers and

willingness of the healthy controls to engage in the author's research. Basal values at the start of the protocols were well matched and within recognised variability for NPD measurements.

6.4.1 Total chloride response is greater with the NPD than the LAPD protocol

It was first important to identify whether the rate of PD perfusion of the LAPD ZCI solution influenced the tCl- measured, as this could impact the overall study results. The tCl- responses were compared by perfusion rate (240 ml/hr *v* 100 ml/hr). Whilst the number of subjects studied was small, especially in the group for the lower-rate (100 ml/hr) group, no significant difference was observed between the tCl- measured at either rate. This suggests that the rate of perfusion does not influence of the amount of chloride perfused.

No statistically significant correlation was identified between the tCl^- response measured by the NPD compared with the LAPD protocol, but the gradient of the 'best fit' line (Figure 6.5) suggested there was almost twice as much chloride secreted by the nasal protocol, and a mean group ratio of nasal to lower airway tCl^- secretion of approximately 1.5:1.

The major variable between the two protocols is the presence or absence of amiloride in the perfusing solutions. It has been previously established from *in vitro* cell studies that blocking apical Na^+ absorption can induce Cl^- movement across the epithelial membrane by hyperpolarising the apical membrane and creating a driving force for Cl^- efflux [228]. This is suggested as a possible explanation for the differences in chloride responses seen, with the absence of amiloride creating an insufficient transmembrane driving force and attenuating Cl^- efflux. As only one measurement (from each protocol) was taken from each individual, it would be essential for future studies to make repeated measurements from healthy volunteers to reduce variability, and to assess both protocols in the lower airway.

6.4.2 Influence of perfusion time on tCl^- response

The author then questioned whether a reduction in CFTR-mediated tCl^- transport was related to the time allowed for tCl^- transport. A comparison of total chloride efflux was assessed by the NPD protocol (after 10 min perfusion) with the LAPD protocol at 5 min, and following an additional 5 min perfusion with ZCI (n=5) to see if further chloride was secreted. This sub-study helped understand whether the LAPD protocol can allow simultaneous active and stimulated chloride transport in 5 min, or whether this protocol failed to allow sufficient time for maximal chloride efflux.

All subjects demonstrated additional chloride efflux in the second 5 min of ZCI perfusion to varying degrees (-2.2 to -15.6 mV), with a mean 'extra' efflux of chloride by -7.1 mV. The comparison between the tCl^- response following 10 min perfusion of NPD and LAPD protocol was incongruous across subjects (n=5), with individuals demonstrating less, more or near equal tCl^- response by the two methods (Figure 6.7). It is therefore difficult to draw conclusions from this underpowered group, and more healthy volunteers would need to be studied in future projects to truly understand the variation of time on the tCl^- response. It is possible therefore that the reduced perfusion time in the LAPD protocol is recording a sub-maximal tCl^- response from the epithelium, and could be a contributing factor in the low tCl^- responses demonstrated in the MDT study. Whilst it is important to minimise perfusate pooling within the airway, it is equally important to fully quantify CFTR function by allowing maximal tCl^- measurements. Previous measurements of tCl^- responses in the lower airway of non-CF subjects have suggested that PD traces plateau following 3 min perfusion of ZCI (Figure 3.12), however the perfusion rate in this study was significantly different at 18 ml/hr compared with those used in this small study [208]. It would be valuable to investigate these parameters further at the current recommended rate and confirm this variability within the lower airways of non-CF individuals in a future research project.

6.5 Conclusions

This chapter has presented a study conducted in healthy volunteers comparing two PD perfusion protocols performed in the nose, and has demonstrated diminished chloride efflux measured by the LAPD protocol. The influence of perfusion rate was considered unlikely to be the cause of this, but the subject numbers were small and the results variable, so interpretation should be made with caution. Secondly, and in support of the first part of the hypothesis, it was demonstrated that extending the perfusion duration of a ZCI solution (LAPD protocol) could produce additional chloride efflux in some subjects, but significant variability was seen in these five people. These results would support the hypothesis that maximal tCl^- response may relate to a crucial need for ENaC blockade (by amiloride) to generate sufficient driving force to maximally move chloride ions through CFTR. It is therefore possible that the current LAPD used may record a diminished tCl^- response and underestimate CFTR function. It would be useful to repeat these studies, in healthy controls, with a focus on comparison of chloride transport within the bronchial epithelium in the presence and absence of amiloride.

Chapter 7: Defining the minimal period between sequential NPDs

7.1 Defining the Benefits and Limitations to Performing Serial NPDs

This chapter will present a study designed to define the minimal period between measuring NPD within the same individual.

Performing serial NPDs in the same individual could be desirable, in particular with regards to serving as a repeated outcome measure in clinical trials. It has been demonstrated in previous chapters of this thesis, and is recognised within the scientific literature, that variability in NPD parameters exists due to (i) the dynamic physiology of the patient's epithelium, and (ii) variability within the measurement technique. By performing more than one measurement in the same individual, it is possible to reduce the error created by intra-subject NPD variability by taking a mean or greatest value and thus increasing statistical power.

Within the MDT, 3 pre-dosing NPDs were performed at different visits, yet only one NPD was performed at each visit following dosing, making it possible that errors related to the natural variability of the test could have attenuated the post-dosing results. NPDs were measured on one occasion in part to minimise patient inconvenience.

The time-limiting factor for repeating NPDs is the duration of action of the drugs within the perfusing solutions – in essence amiloride and isoprenaline, and the time taken for the epithelium to return to its electrophysiological baseline. To date, no evidence-based guidance exists to suggest an interval between serial NPDs being performed on the same individual to ensure that the baseline has returned. Within the MDT, a 4-hour interval was used based on previous in-house data (unpublished), as this period had been shown to have no effect on subsequent PD measurements. This study aimed to define this interval more precisely, and optimise the throughput of patients in future trials.

7.2 Restoration of Airway Epithelium Equilibrium

The ideal situation is for the initial basal measurement, in response to a Ringer's solution, to have returned to its baseline at the start of subsequent NPD. This requires the Na^+ and Cl^- to have returned to the starting equilibrium, and necessitates cessation of the amiloride blockade on ENaC and restoration of innate CFTR function with the normalisation of levels of epithelial cAMP. In CF, the response to amiloride represents the change in epithelial potential difference in its near-entirety, with minimal changes occasionally being seen in response to the chloride-free solutions. It was therefore decided by the author to investigate the duration of action of amiloride on the nasal epithelium following an initial NPD.

7.2.1 Duration of action of amiloride on the airway epithelium

It has been demonstrated that amiloride has a short duration of action on the mucosal surface owing to both rapid absorption and rapid reversal from ENaC block [200], which prevented its use clinically as a nebulised agent to improve mucociliary clearance or lung disease in CF [229]. A model measuring drug concentration following the aerosolisation of amiloride (6×10^{-3} M) onto the ASL of awake sheep reported a rapid disappearance of the drug within 30 minutes and approximated the half-life to 10.5 minutes [230].

7.2.2 Repeated NPD following nebulised administration of amiloride

Consecutive NPD following amiloride administration has been previously measured by the Gene Therapy research group [231]. Middleton *et al* compared serial NPD measurements in 8 CF patients following nasal nebulised amiloride (1 mmol/l) or placebo (0.9% saline); Middleton used an ultrasonic nebuliser and

delivered the solutions for a total of 5 min (delivering a total volume of 4-4.5 ml), measuring the basal NPD by inserting a modified Foley catheter at baseline and at 5, 15, 30, 45 and 60 minutes. Whilst the basal PD slowly returned to baseline (Figure 7.1), Middleton reported that at 60 minutes the PD was still significantly reduced following amiloride administration compared with the initial PD and to that of the saline group. This work however does not replicate the NPD method or concentration of amiloride perfused onto the nasal epithelium.

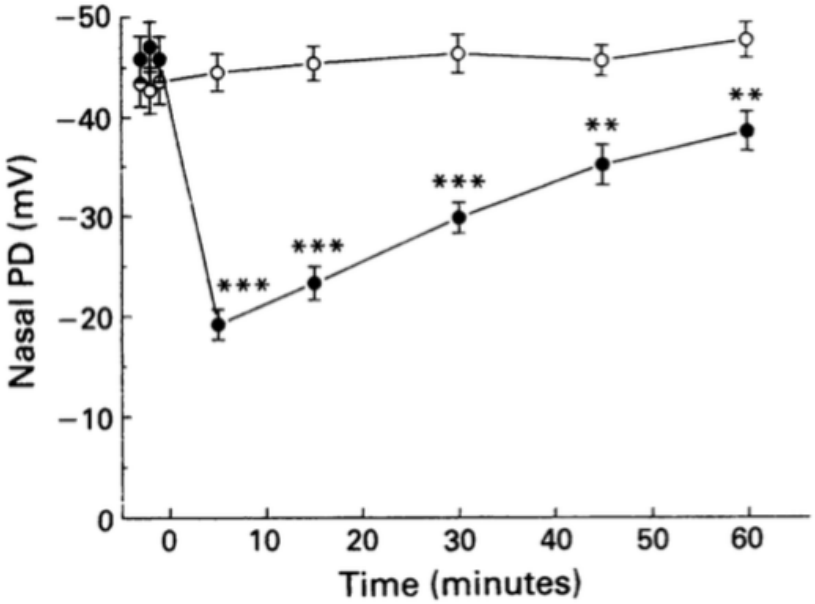


Figure 7.1. Graph showing serial NPD measurements in CF patients (n=8) following administration of amiloride (black circles) or 0.9% saline (white circles); *** p<0.001; **p<0.01 (compared to saline). Reproduced from Middleton *et al,* 1993 [231].

7.3 Evaluating the minimal time interval to perform serial NPD

7.3.1 Study hypothesis and aim

Based on the data from Middleton *et al* [231], I have hypothesised that it will take at least 60 min for the basal PD to return following NPD Ringer's and RA perfusion, and would approximate this duration to 90 min by extrapolating their

amiloride treatment line (Figure 7.1). The aim of this study was to calculate the period of time that amiloride blocks ENaC and depolarises the nasal airway epithelium in patients with CF. This would provide important information, which could be used to optimise the design of future CF trials and/ or clinic visits.

7.3.2 Subject recruitment

Adult (>18 years) CF patients were recruited from the CF ward at the RBH having been identified by the clinical CF team. 8 patients were expected to be required for this study, based on previous in-house data. Patients were approached when deemed clinically stable and all interruptions to usual CF inpatient care (e.g physiotherapy sessions and intravenous antibiotic administration) were avoided.

7.3.3 Study exclusion criteria

Patients were excluded if they had significant nasal polyps or sinus disease or current nasal symptoms, were requiring oxygen therapy or were taking oral amiloride therapy.

7.3.4 Ethical approval, consent and documentation

An ethics committee (NRES, London – Brent Committee, Ref: 13/LO/0391) approved the study and all patients provided full informed consent. Patients' medical records and current medication chart were reviewed to ensure suitability for the study. Each patient was given a unique identifier to maintain anonymity, each being prefixed 'AMIL' and followed by a number, e.g. AMIL001.

7.3.5 Infection control

Patients were investigated in the CRF at the RBH, and strict infection control procedures were followed in accordance with the hospital's policies (as outlined in Chapter 2, section 2.4.7).

7.3.6 Nasal PD set-up

A NPD circuit was setup as described in chapter 3, but only 2 syringes of perfusing solution were required, namely Ringer's and Ringer's + amiloride (RA) solutions;.

7.3.7 Positioning of the nasal catheter

The most negative baseline PD was identified from both nostrils and the catheter fixed at this position – this position was used for all subsequent PD measurements.

Patients could opt to have the NPD catheter removed or left in situ between serial PD measurements; for both situations, the catheter was immediately flushed with approximately 2 ml of Ringer's solution (the volume of the catheter lumen, to expel all RA solution from the channel and prevent contamination on the subsequent PD phase, but not 'wash' away already perfused amiloride from the mucosa).

7.3.8 Perfusion protocol for NPD solutions

Ringer's solution was perfused at 4 ml/min until a stable basal PD was obtained and this value recorded; this starting basal PD was the target for subsequent PDs. RA solution was next perfused at 4 ml/min for a minimum of 3 minutes and until

a stable PD was obtained when perfusion was stopped.

A 30-minute interval was observed before a further NPD was performed. Shortly prior to restarting an NPD, the circuit was tested by checking the skin PD and the catheter was reinserted (if necessary); if the catheter was left in situ, it was not possible to check the circuit.

Perfusion was restarted with Ringer's solution until a stable PD was obtained – the aim was to achieve at least the initial basal PD, and NPDs would be serially performed until such a time. If the initial basal PD was reached, RA was re-perfused until stable which completed the protocol; otherwise perfusion of Ringer's stopped and a further 30 minute interval observed, following which Ringer's would be again perfused. This protocol is outlined in Figure 7.2.

7.3.9 NPD analysis

All NPD traces were converted from a text file into an Excel file, and reproduced graphically. All traces were analysed in Microsoft Excel by MDW using the data analysis method previously described.

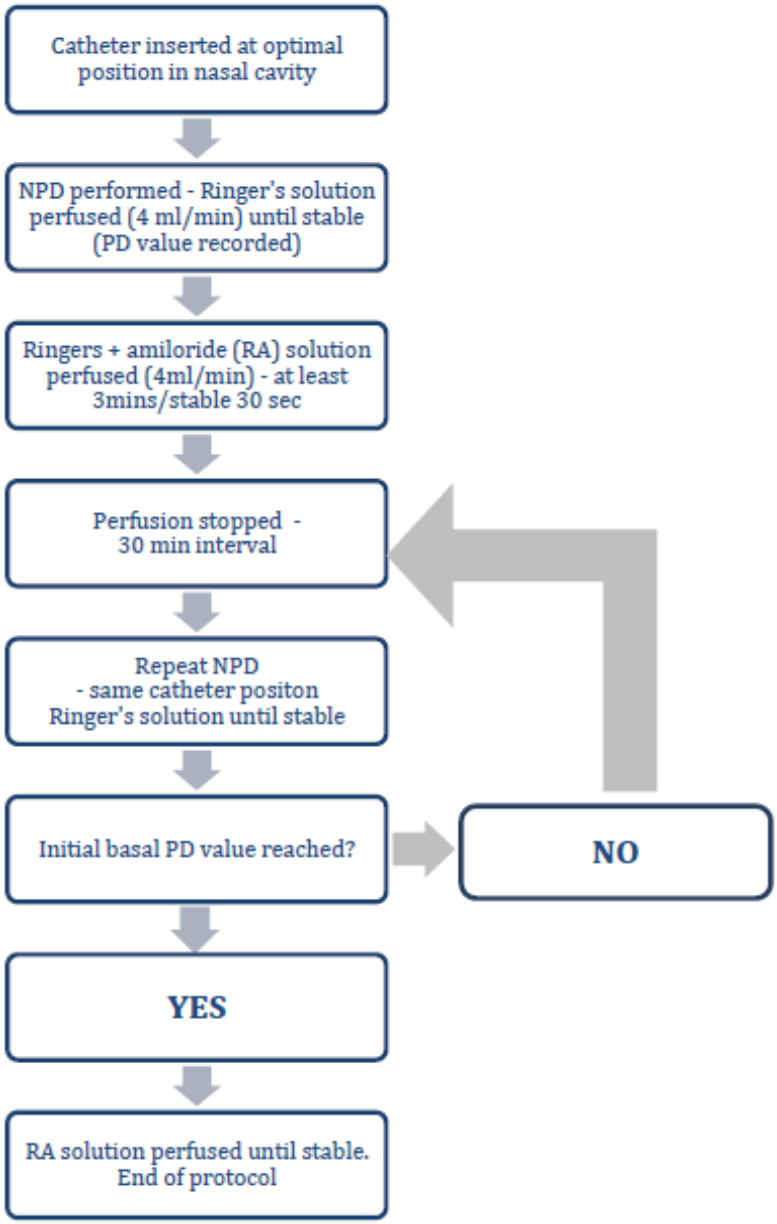


Figure 7.2. Schematic overview of the protocol for performing serial NPDs

7.4 Results

7.4.1 Subject enrolment and demographics

In total, 9 CF patients were recruited for this study. 1 patient (AMIL005) was excluded owing to an absent amiloride response of undetermined reason (the

patient denied current nasal symptoms, though did have an upper respiratory tract infections 2 weeks earlier). The results from the 8 subjects will be presented. Individual and group patient characteristics are shown in and summarised Table 7.1

Subject ID (AMIL-)	Age yr	Sex	Mutation Arm 1	Mutation Arm 2	FEV_1 (%)	PI / PS	Catheter Position (cm)	Catheter Removed?
01	38	M	F508del	F508del	35	PI	6.0	NO
02	23	M	F508del	1609delCA	56	PI	6.0	NO
03	21	M	F508del	F508del	61	PI	7.75	YES
04	27	M	F508del	F508del	50	PI	8.0	YES ‡
06	27	F	F508del	F508del	54	PI	6.0	NO
07	20	M	F508del	F508del	46	PI	7.5	NO
08	19	F	F508del	F508del	55	PI	7.0	YES
09	18	F	F508del	F508del	84	PI	7.0	NO
Mean (SD)	25 (6.7)		-	-	54 (13)	-	-	

Table 7.1. Basic demographics and genotypes of individual study patients. PS – Pancreatic Sufficient; PI – Pancreatic Insufficient. Catheter removed following first NPD then reinserted. ‡Patient had catheter between 1st and 2nd NPD, but remained in situ for the 3rd (final) NPD.

7.4.2 Catheter removal between serial NPDs

In total, 3 patients requested to have the NPD catheter removed/reinserted between serials NPDs, while at the subject's request, one of these patients had the catheter left in place between the 2nd and 3rd (final) NPD; the other 5 patients all had the catheter left in situ between measurements.

To address whether the removal and reinsertion of the NPD catheter created a difference in PD results from those where the catheter remained in situ, the percentage change between the initial and final basal PD were compared. It is

clear from Figure 7.3 that more variability is seen in the change from initial to final basal PD in the 3 subjects where the catheter was removed compared with the 5 subjects left in situ. This may suggest a limitation in the final data regarding the reproducibility of basal measurement following catheter removal and re-insertion. For this reason, all subsequent results will denote the subjects having the catheter removed to accompany data interpretation (these subjects were AMIL-03, AMIL-04 and AMIL-08).

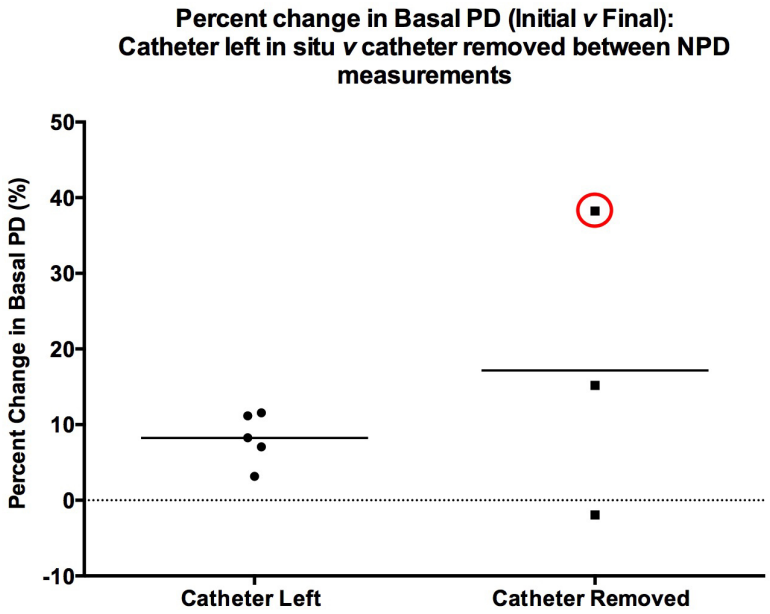


Figure 7.3. Graph displaying individual subject percentage change between initial and final NPD measurements with nasal catheter left in situ or removed. The subject circled red had the catheter removed following the initial NPD but left in situ for the final NPD.

7.4.3 Summary of individual NPD traces

Summaries of the PD measured at each perfusion phase from the 8 subjects are shown in Figure 7.4, with the first post-amiloride Ringer's (time point C/Ringers T1) commencing perfusion at a mean (range) time of 30 min (24-36), and the second Ringer's (time point D/Ringers T2) at 61 min (58-65). Figure 7.5 displays the same data, but compares each change in PD following perfusion to the change from their baseline basal value.

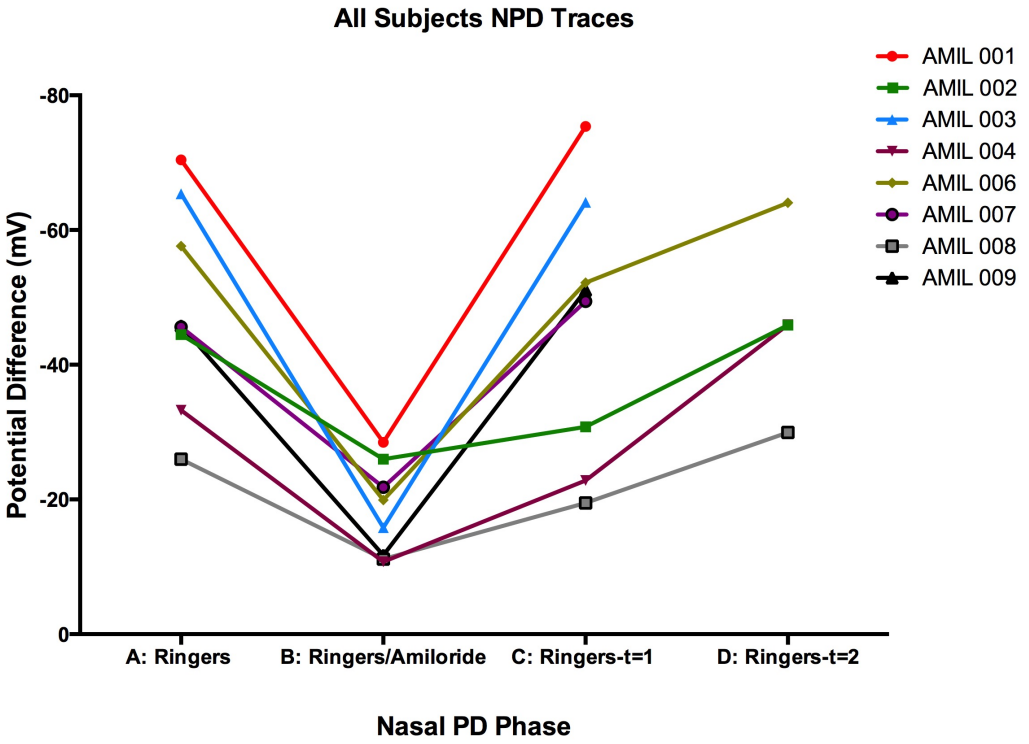


Figure 7.4. Summary of individual NPDs at (A) baseline, (B) in response to Ringer's/amiloride solution, and subsequent Ringer's perfusion – (C) t=1: at 30 min, and (D) t=2: 60 min. The following subjects had their catheters removed and re-inserted: AMIL-03, AMIL-04 and AMIL-08.

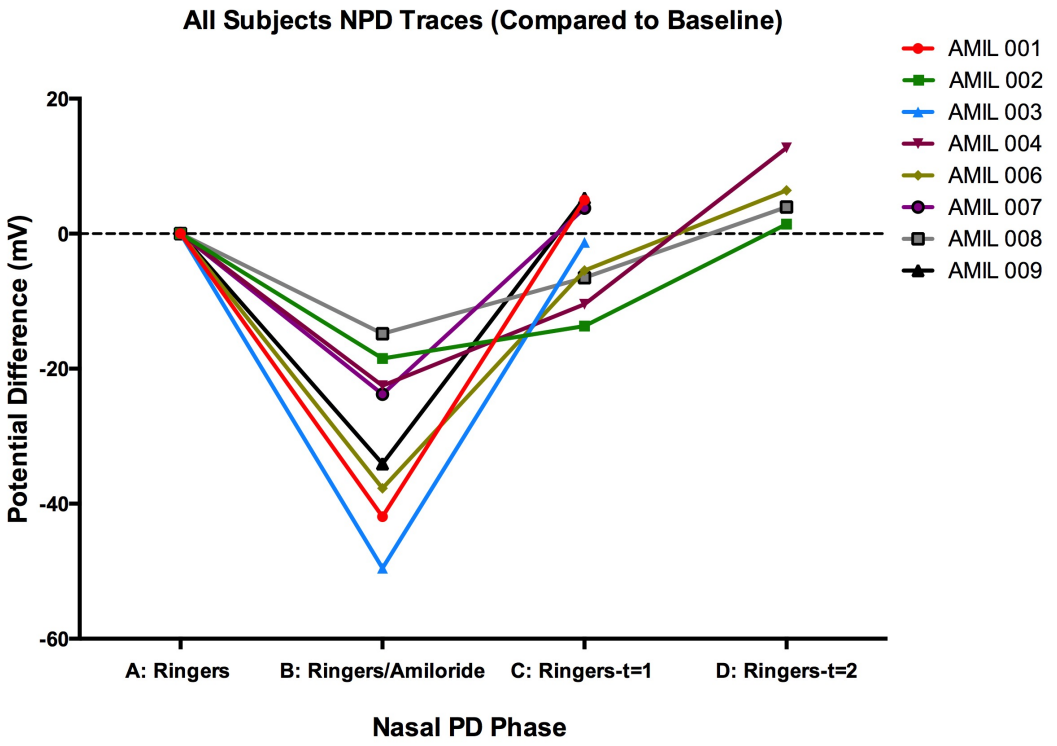


Figure 7.5. Summary of individual NPDs compared to initial basal PD at (A) baseline, (B) in response to Ringer's/amiloride solution, and subsequent Ringer's perfusion – (C) t=1: at 30 min, and (D) t=2: 60 min. The following subjects had their catheters removed and re-inserted: AMIL-03, AMIL-04 and AMIL-08.

7.4.4 Time to return of initial basal PD following amiloride perfusion

Following perfusion with RA, the basal PD had returned to the original basal measurement in 4/8 (50%) patients after 30 minutes, and in all 8 patients after 60 minutes. In patients having the catheter removed, 2/3 returned to baseline by 30 min. In the 4 patients where the basal value had not returned after 30 minutes, the percentage of reversal of the basal potential (following amiloride) for each patient was 26%, 54%, 86% and 56%.

7.4.5 Change in basal PD following Ringers/amiloride perfusion

All patients except one had a small increase (more negative) in basal PD following RA perfusion compared with their original value, with a mean (SD) increase of -4.7 mV (4.1) (p=0.01, paired t-test). Patient AMIL-03 had a final Ringer's value 1.3 mV lower (less negative) than their starting basal value; this was a patient having their catheter removed/re-inserted, with their basal PD returning by 30 min. At the time of performing the PD, it had appeared that the basal value had returned, but during data analysis it was found to be slightly lower. The change in basal PD following perfusion with RA is shown in Figure 7.6, with subjects having their catheter removed/re-inserted highlighted in red.

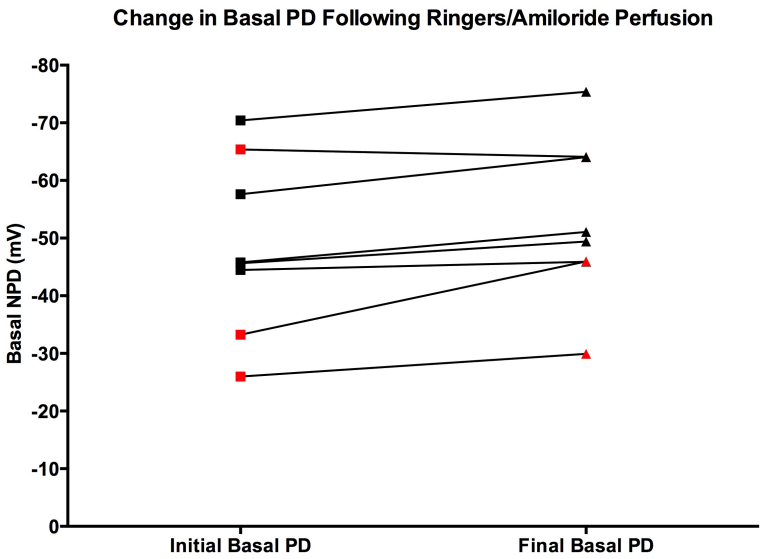


Figure 7.6. Change in basal PD before and at the final NPD following perfusion with RA solution. Data points in red denote the catheter was removed/re-inserted.

7.4.6 Second perfusion with Ringers/amiloride

7.4.6.1 Difference in airway PD following second RA perfusion

Once the basal PD had returned to at least the initial baseline, RA was perfused again. Whilst statistically there was no difference between the initial and final PD following either perfusion with RA (p=0.80, paired t-test), Figure 7.7 demonstrates variability in the subsequent post-RA PD, with some patients failing to reach their starting post-RA measurement.

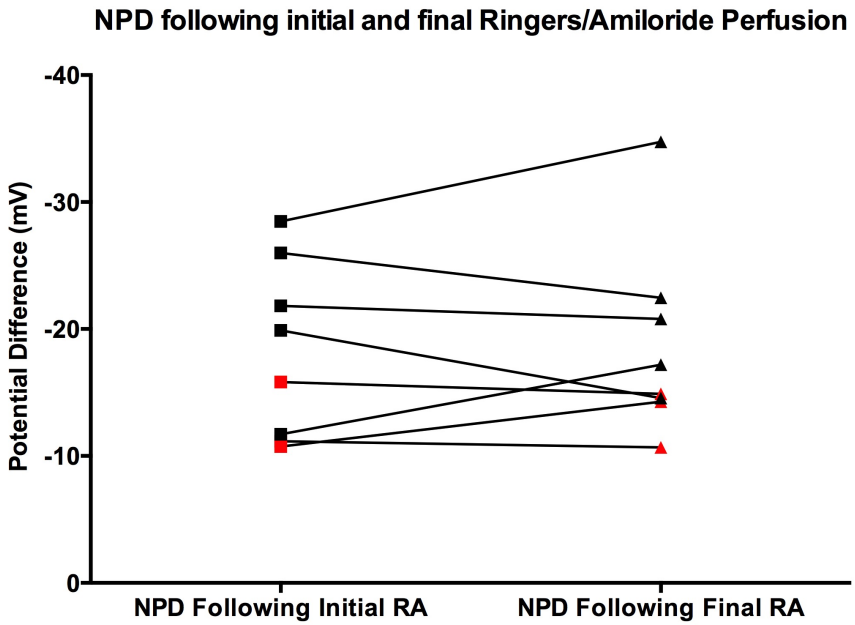


Figure 7.7. Graph showing the initial PD and final PD following perfusion with RA (n=8). Data points in red denote the catheter was removed/re-inserted.

7.4.6.2 Difference in dAMIL following second RA perfusion

The mean (SD) difference in response to the second perfusion of RA was 4.0 mV (4.0) (p=0.04, paired t-test). Two subjects has an increased dAMIL by 9.2 and 9.3 mV on the second perfusion compared with the first – the former was noted to have an increase in their basal PD of -12.7 mV, whereas the latter had an essentially unchanged basal PD (-1.4 mV). These changes are summarised in Figure 7.8.

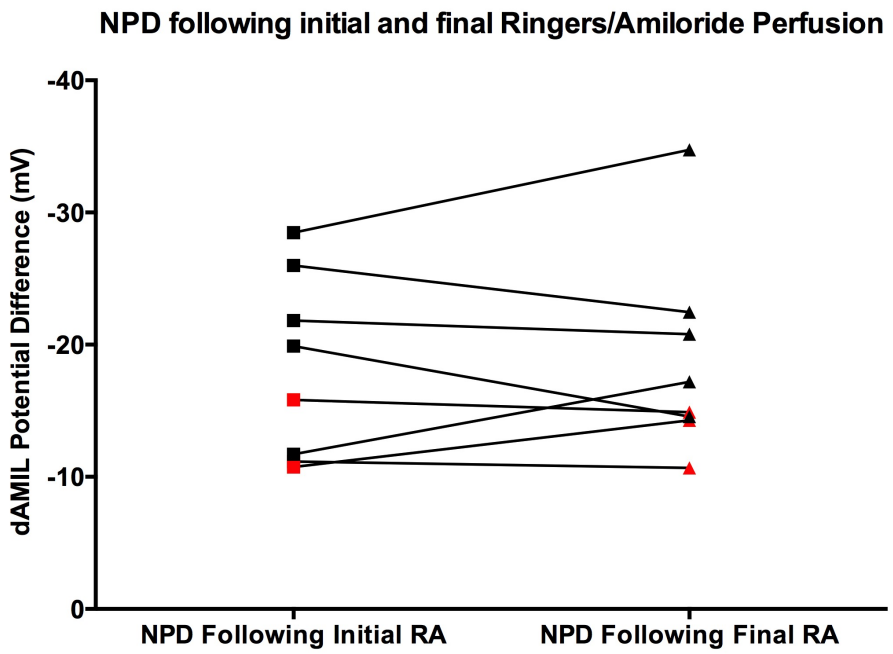


Figure 7.8. Graph showing the change in dAmil at baseline and at final perfusion (n=8). Data points in red denote the catheter was removed/re-inserted.

7.4.7 Time to achieve PD stability

When comparing the time taken to achieve stability with perfusion of RA at the initial and final time points, wide variation was seen throughout the group (Table 7.2); no relationship to the catheter being left in situ or removed was seen.

Duration for RA phase to reach stability (s)	Initial NPD	Final NPD	Change
Mean (SD)	388 (146)	377 (121)	-11 (187)
Range	240 – 652	248 – 534	-292 – 256
p-value (paired t-test)	p=0.87		

Table 7.2. Table summarising the mean, SD and range for the duration of Ringers/Amiloride perfusion to achieve stability at the initial and final NPD, and the change. P-value shown is calculated from a paired t-test.

No correlation was identified between dAMIL and the time taken to reach stability following the initial RA perfusion (r=-0.09, p=0.84, Pearson correlation) or the final RA perfusion (r=0.47, p=0.24); similarly the change from the initial to final dAMIL measurements with the change in time to achieve stability showed no correlation with each other (r=-0.01, p=0.99, Pearson correlation).

7.5 Discussion

This study was designed to define the minimum time period between performing serial NPDs and to provide confidence to the observer that changes in epithelial ion transport following one NPD have returned to baseline at the start of the next. It focused on the sodium component of the NPD, as this produces the largest change in PD in the CF epithelium.

7.5.1 Minimal interval between serial NPDs

The results demonstrated that in 8 patients with CF, the basal PD returned within 60 min for all patients, with 4 patients recording normalisation of epithelial PD within 30 min. The author concludes that for any future trials requiring NPD to be taken sequentially, the sodium transport of the CF

epithelium will have returned to baseline 1 hr after amiloride perfusion has discontinued, and a further NPD can be reliably performed.

In a similar study, Middleton *et al* [231] found that the basal PD had not fully returned after 60 min following administration of nebulised amiloride solution (1 mmol/l) onto the nasal mucosa. The study presented within this chapter has replicated NPD, with amiloride being perfused onto the mucosa in Ringer's solution; in addition Ringer's solution was perfused to measure subsequent basal PD, which itself may have 'washed' amiloride from the nasal mucosa.

One significant variable between the subjects studied is whether the NPD catheter was left in situ or removed. Previous work has demonstrated that removal/reinsertion of the catheter does not affect the PD measurement. It is possible that within this study the maximal site of amiloride perfusion and the site of basal PD measurement could differ following removal and reinsertion of the catheter. It is clear that significant variability in basal PD measurements is seen in the 3 patients to whom this applies, and the interpretation of their results could under- or overestimate the true duration of action of amiloride.

This study did not fully replicate the entire NPD protocol, having not assessed the influence on perfusing ZC solutions containing amiloride and whether the extended exposure (a further 10 min) of the epithelium to amiloride alters the time taken to return to baseline.

Furthermore, this study did not address any influence of the ZC and isoprenaline components of the NPD protocol on epithelial PD. It would be desirable to study this, as (i) the author has regularly observed further decreases (more positive) in epithelial potential following ZCA perfusion, which is possibly related to ion movement other than sodium and chloride across the epithelium; and (ii) the lasting effects of chloride-free and isoprenaline solutions in studies on Cl^- homeostasis. It is not possible to determine the latter in CF patients owing to the absence of Cl^- efflux, and so future studies should address these questions in healthy (non-CF) controls.

7.5.2 Dichotomisation of CF responders to amiloride

There is an apparent dichotomisation in sodium transport of the CF patients studied, with some patients measuring a higher basal PD (approximated to -50 to – 70 mV) and following an appropriately large dAmil response their basal PD returns quickly (within 30 min), and a second group having a lower basal PD at baseline (approximated -25 to -50 mV) and following a smaller dAmil response taking up to 60 min for the original basal PD to return.

It is possible from the small number of CF patients studied that differences in ENaC responses may be present, and it could be extrapolated to the relationships between airway epithelial sodium transport and disease severity.
ENaC is formed of 3 subunits – α, β and γ, with the lung epithelium being likely composed of two α, one β and one γ subunits, but it is possible that different forms are expressed in different regions of the lung composed of variations in the subunits, perhaps with the inclusion of a δ subunit [232]. The open probability and channel kinetics of ENaC have been shown in rat epithelium to differ depending on the subunit composition [233]. ENaC that is formed solely by α and β subunits exhibited a very high open probability and relative insensitivity to amiloride, in direct contrast to those including a γ subunit which displayed a high amiloride potency [234]. It could be hypothesised that the variations seen from these *in vivo* PD measurements could therefore relate to undetermined variations in ENaC structure or function.

However, few conclusions should be drawn until this has been repeated in a larger number of patients. It would be useful to repeat this work to incorporate disease severity, owing to the close links between sodium transport and clinical outcome.

7.5.3 Change in basal PD following amiloride perfusion

The final basal PD recorded was more negative in 7/8 patients, with a statistically significant mean change of -4.7 mV compared with baseline; there was also a significant mean change in dAmil of 4.0 mV on second perfusion which is likely a reflection of this change in basal measurement. It is possible that intra-subject variability in PD measurement could account for these findings, but it may be a reflection that the epithelial ion homeostasis has not yet been fully restored.

If this study were to be repeated, it would be instructive to measure the second basal PD following RA perfusion at a second time point following return of the initial PD to understand whether this value remains higher or falls over time. It would be prudent to repeat this in a greater number of patients, and leave the catheter *in situ* throughout the entire duration of study to minimise error.

7.6 Conclusions

This study aimed to identify an interval for performing serial NPDs within the same study subject. It was not anticipated that patients would request the nasal catheter was removed between measurements and this may have created variability within the results. Nonetheless, these data suggest that basal PD will return in CF patients 60 min after terminating RA perfusion, thus rejecting the original hypothesis, and suggesting repeated measurements can to be taken over a short timeframe for clinical studies. The author however suggests it is essential to repeat this work and incorporate ZCA and ZCAI perfusion before taking this work into clinical and research practice.

Chapter 8: Overall Summary and Conclusions

This thesis has explored the pathophysiology of cystic fibrosis and the underlying defect, and presented the Multidose Trial of repeated application of *CFTR* gene therapy as a proof of concept to ameliorate the epithelial defect and assess clinical improvements in CF lung disease. The thesis has focused largely on changes in ion transport within the upper and lower airway epithelium, to understand its behaviour and relationship to measurements of lung function at baseline, and its changes in response to treatment. Throughout this thesis, confounding variables and limitations to potential difference have been discussed, and studies are reported, performed by the author, to help understand the relationship between the epithelium at both sites, and to optimise performing and interpreting PD results.

Each chapter has presented its own results, discussions and individual conclusions. This chapter will provide some overarching thoughts and conclusions from this thesis, and will discuss the possible future of gene therapy and restoring CFTR function.

8.1 Nebulised pGM169/GL67A as a treatment for cystic fibrosis

The Multidose Trial has demonstrated proof of concept that repeated nebulised dosing, every 28 ±5 days, of 5 ml of pGM169/GL67A can lead to a small but statistically significant change in lung function.

The study demonstrated the difficulties in recruiting patients into a trial lasting at least 12 months and with frequent study visits, particularly from a cohort of children and young adults who have less severe disease and who are therefore in education or employment. The motivation of the patients and the trial staff helped complete the study with 85% retention of patients. The overall results demonstrate stability in FEV_1 in response to active treatment, rejecting the original hypothesis of this thesis, an improvement in lung function; this itself is a

respectable outcome for a progressive disease, however the longer-term treatment effect needs to be established. A surprising dichotomisation in treatment effect, with encouraging trends towards improvement in FEV_1 in patients with more severe baseline lung function, was demonstrated. Further, the efficacy was small compared with those from available CF symptomatic treatments and ivacaftor. It is, therefore, likely that optimisation in the dosing of pGM169/GL67A is required before considering it as a licensed therapy. Furthermore, this study did not demonstrate that improvements in lung function were associated with a reduction in pulmonary exacerbations (number of antibiotic courses), although a *post hoc* analysis was not made in the subgroup demonstrating the largest treatment effect. Pulmonary exacerbations are a huge burden of morbidity in CF patients, and successful treatment must demonstrate their reduction, with a resultant improvement in quality of life. It is possible that the current dose of gene therapy is insufficient to demonstrate a reduction in pulmonary exacerbations. It is worth noting that pulmonary exacerbations were only indirectly captured in the MDT, and that therefore under- or over-estimation of the true incidence may have occurred. Future studies should use a validated method to record exacerbations, to appreciate the true treatment effect.

A significant strength of this study was the low missed dosing rate of the IMP, with 93.5% of active doses delivered to patients. This, in part, is likely to relate to the monthly administrations being undertaken in a dedicated research facility. This has obvious advantages over long-term studies, where compliance with research medication administered at home wanes [235]. Despite this, the effect of missing one or more doses of gene therapy is unknown, and may have contributed to a smaller observed effect if consistent CFTR expression is required for clinical benefit. It may be, therefore, that the study was too 'lenient' towards patients having interrupted therapy, and that future studies should mandate a maximal interval between doses rather than an overall 'missed dose' threshold.

Within the current CF therapeutics pipeline are several phase II and III clinical trials of CFTR modulators, with drugs and genetic approaches targeting specific *CFTR* genotypes. Thus, a novel approach to 're-programme' *CFTR* mRNA using spliceosome-mediated RNA trans-splicing (SMaRT) commenced a phase Ib study in 2015. mRNA re-programming, in contrast to the study reported here, does not require the formulation to reach the nucleus to exert its effect. However, both the therapeutic RNA and any repaired mRNA are likely to have low stability creating other potential problems. Finally, it is important to note the cell membrane and extracellular barriers that will be common with conventional gene therapy approaches. Gene therapy is running parallel to these trials, but offers the opportunity to provide a universal treatment for all CF patients rather than being mutation-specific. Furthermore, adherence to timely, complex treatment regimens in CF is poor [236], and therefore a once monthly treatment with gene therapy could markedly reduce treatment burden and have a positive effect on quality of life.

8.2 Efficacy of pGM169 *CFTR* gene transfer

Mechanistic outcomes assessing effective gene transfer were used in the study, but were opportunistic and underpowered. The assays used were nasal and lower airway potential difference to measure *in vivo* CFTR function, and quantification of vector-specific DNA and mRNA.

No treatment difference was measured in the upper airway between patients treated with gene therapy or placebo, however a significant difference was seen from the lower airway in the active treatment group. Furthermore several patients from the active group demonstrated electrophysiological evidence suggestive of *de novo* CFTR function in both the upper and lower airway. Molecular evidence supported gene transfer into both nasal and lower airway epithelia, with quantifiable vector-specific DNA being measured in 14/15 bronchial wall samples and 15/16 nasal brushing samples, however no pGM169-mRNA was measured at either site. As the mRNA assay has poor sensitivity and

is affected by high-levels of total RNA[210], this finding is disappointing but not unexpected. Further, the MDT used 0.9% saline as a placebo, choosing not to use a *CFTR*-deleted plasmid/liposome complex because of the absence of safety data of the pGM169/GL67A complex, to allow comparison with the natural progression of CF airways disease and also for financial reasons. Thus, there is a formal possibility that the observed effects on lung function could be a non-specific effect of the gene therapy complex, and may related to its interaction with the innate immune response and bacterial killing. Future work may involve improvements to the sensitivity of the mRNA assay, and assessing the safety of a *CFTR*-deleted or CFTR mutated plasmid/liposome complex to use as a placebo.

It has been discussed that variability in PD changes in general may relate to both variability within the PD technique and the inherent fluctuations in epithelial physiology. Further factors specific to this work include patchy gene uptake within the epithelium and a discrepancy between the maximal site of gene delivery and the site of PD measurement. It is also possible that, in some patients, maximal gene expression was shorter than that between dosing and PD (NPD: 28 ±5 days; LAPD: 27-36 days) and therefore maximal correction was missed. However, assessment of the day 14 measurements did not show important differences to those taken at day 28. It is possible that changes in the lower airway PD were underestimated, as the absence of amiloride in the perfusate has been shown (chapter 6) to reduce epithelial chloride transport. It would be advantageous for studies to evaluate the effect of adding amiloride to the perfusate measuring LAPD chloride responses in healthy volunteers, to determine whether future gene therapy trials could augment measurements of chloride efflux.

A statistical relationship between the upper and lower airway PD was not clearly established in this thesis. NPD is used as a convenient surrogate for PD measurements of the lower airway, and this work could not further the understanding or behaviour of one in relation to another. Future studies should not conclude changes from the lower airway solely based on electrophysiological

changes in the nose, and therefore NPD should not replace the role of LAPD outcome measurements for future studies.

To reliably use NPD as a trial outcome measure, the PD needs to differentiate a treatment effect from intra-subject variability. Physiological variability reported to influence NPD includes epithelial hypoxia, mucosal dryness and the response to exercise [237-239]. Factors contributing to physiological variability should clearly be minimised in future studies but are not well worked up and require further attention. The author also proposes duplicate measurements should be taken, and for future studies the author would support measuring NPD from both treated nostrils. It remains unanswered what the optimal PD value is: should PD measurements take a mean value, or the most negative? Averaging NPD results from both nostrils reduces the variability between both nostrils and repeated measurements [221]. However a retrospective study concluded this might underestimate CFTR function [240]. Taking measurements from the site of most negative PD however is likely to reflect sodium transport more than CFTR, and may miss a maximal chloride response [241]. It is the author's opinion that PD measurements are a useful tool, and despite their inherent variability, should remain a clinical endpoint in adequately powered future studies.

8.3 The relationship between epithelial ions and disease phenotype

This thesis has discussed the importance of CFTR-mediated chloride transport across the airway membrane. However data within this thesis and within the CF scientific literature have identified correlations between indices of airway sodium transport with lung function. This thesis has identified a possible dichotomised NPD response in CF patients having serial NPD measurements taken after amiloride perfusion, with some individuals being shown to have a more potent response to amiloride. It is therefore hypothesised that variations exist in the structure, expression or regulation of ENaC leading to differences in the CF airway ion and water transport, and severity of airway disease. If this

study were to be repeated, it would be useful to calculate a larger sample size based on the data presented, with each CF patient having measurements taken on at least 2 occasions. As the carrier rate of *ENaC* polymorphisms are estimated as 9% of the general population [242, 243], it would be useful to check for these in the patients, to determine whether direct ENaC function is further inhibited due to this variation in addition to the effects from CFTR.

The author has however highlighted a few individual cases of CF patients with a mild disease phenotype, each having a preserved 'chloride' response. When the term 'chloride response' is used, it refers to a hyperpolarisation of the airway epithelium in response to the luminal surface becoming more negative as negatively charged ions exit the cell membrane, predominately through CFTR. In addition to chloride, it is well recognised that CFTR also allows movement of bicarbonate ions (HCO_3^-), of which there may be a reduced concentration on the CF airway epithelial surface [244] . A failure to secrete HCO_3^- onto the epithelial surface will result in a more acidic pH of the airway surface liquid, which is known to impair the function of innate antimicrobial molecules and reduce bacterial killing [28]. It has been hypothesised that a lack of bicarbonate reduces the normal expansion of mucin molecules, rendering mucus more viscous and impairing mucociliary clearance [8]. Thus, patients demonstrating a partial chloride hyperpolarisation, may have some preserved HCO_3^- efflux as well, lessening the pathological effects on the airway mucosa. Measurements of airway short-circuit currents have demonstrated epithelial hyperpolarisation during stimulation of CFTR (by the c-AMP stimulant forskolin), that are terminated by carbonic anhydrase inhibitors (which block the formation of bicarbonate) [244]. Thus, the addition of bicarbonate to CFTR knockout mice ileal epithelium restores mucus properties [245]. However, direct bicarbonate measurements are difficult to make in man, owing to its rapid dissociation in the presence of air.

The author suggests it would be advantageous for future studies to use NPD to measure *in vivo* bicarbonate transport, to help evaluate whether epithelial changes are related to chloride, bicarbonate, or both, and whether patients with

preserved bicarbonate transport have a milder phenotype and survival advantage. The author had intended to incorporate the carbonic anhydrase inhibitor acetazolaminde into a NPD protocol, and seek ethical approval for this project during the time course of this PhD – however this did not fit within the time frame of the PhD.

8.4 The potential for correcting CFTR function

In 2013, a close friend's nephew was born with CF and his uncle asked the author what his future would entail. It was comforting to tell him that the modern era of CF medicine is rapidly progressing with the aim no longer to correct disease sequelae, but to correct the underlying defect itself. Numerous investigational products are now leaving the laboratory and being studied in clinical trials, with some entering clinical practice, and that a near-normal life expectancy is a realistic possibility.

The Multidose Trial demonstrated stability in lung function which itself is encouraging to patients, their families and carers. Since I left the UKCFGTC, future trials of non-viral pGM169/GL67A gene therapy have been planned with the proposal to optimise the treatment dose and dosing interval, and as well as investigating additional benefits of using a CFTR potentiator to augment transgene CFTR function. In parallel, the Consortium is preparing for the first-in-man F/HN-pseudotyped lentivirus vector transfer of *CFTR* into the CF epithelium [163].

After over a decade of work, the Consortium can proudly report a major step along the path of correcting cystic fibrosis at the cellular level for all patients, and provide optimism that the next step could be even more successful.

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10. Appendices

Appendix A – Gene therapy Multidose Trial adverse events grading table

Multi-Dose Trial of Gene Therapy in Cystic Fibrosis Patients
GRADING TABLE FOR ADVERSE EVENTS

Guidance Notes for using the grading table:

1. Baseline measurements are defined as those immediately prior to dosing.

2. * **General deterioration in respiratory status** – defined by 2 or more of the parameters tabulated.
   - The severity is determined by the MOST severe individual parameter.
   - Each individual abnormal parameter must be recorded on InForm database.

3. ^ **Flu-like symptoms** – defined by 2 or more of the parameters tabulated.
   - The severity is determined by the MOST severe individual parameter.
   - Each individual abnormal parameter must be recorded on InForm database.

4. **Activities of Daily Living (ADL):**
   a. **Instrumental ADL** refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc
   b. **Self care ADL** refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden

5. Abbreviations
   a. ULN – Upper Limit of Normal
   b. IV – Intravenous
   c. Hrs – Hours
   d. Mins – Minutes

6. **Guidance on assignment of adverse events causation to aid cross-site consistency in recording/ assigning causality** and in correct documentation on the CRF adverse events worksheets

    i. **Isolated upper respiratory tract symptoms:**
        a. should be considered separately to chest-related symptoms
        b. may have different causality assigned in nasal dosing patients than non-nasal

    ii. **Timing of chest-related/ general respiratory symptoms:**
        a. After dose 1:
            i. Day of dosing/ D1= 5
            ii. Days 2/3=4
            iii. Days 4-7 = 3
            iv. Beyond 1st week= 2

        b. After dose 2-12 e.g. cough, sputum, wheeze etc will influence grading of causality:
            i. Day of dosing/ D1= 5
            ii. Days 2/3=4
            iii. Days 4 and beyond= 3
            iv. Grading chest symptoms with a 2 will almost never be appropriate (exception to this might be a patient who has missed 3 consecutive doses and presents for dosing with very short history of recent increase in symptoms)

    iii. **Timing of systemic inflammatory response** (fever, headache, lethargy etc):
        a. Day of dosing/ D1= 5
        b. Days 2/3=4
        c. Days 4-7 = 3
        d. Beyond D7, 2 might be appropriate, especially if clear viral symptoms

iv. Any **causality grade may be increased if that patient** repeatedly reports same symptoms/ constellation of symptoms at same time with each dose e.g. increased cough/ sputum on day 2 may be graded 5 if pattern clearly seen

v. **Grade 1 should only be given under the following circumstances**:
   a. Patient has not yet received 1st dose
   b. No possible theoretical reason for link with this organ, eg. broken leg; post-surgical complication
   c. Possible tenuous link, but patient has this complaint repeatedly pre-dating the trial and frequency, severity etc has not changed eg. DIOS. (new DIOS could be caused by inflammatory response to ingested product or by dehydration resulting from flu-like AEs). If in doubt, score 2.

vi. **Grouping symptoms**:
   a. Only do this if start at same time and if relationship/ causality is similar for both or more symptoms eg.
      i. OK for: fever, headache, lethargy starting D1
      ii. Not OK for cough, abdo pain, diarrhoea starting D2
   b. Symptoms that start as a constellation
      i. should be considered as resolved after the last symptom has resolved
      ii. should be graded according to the most severe component

vii. Causality for **new microbiological growths or new CT changes** (e.g. worsening of mucus plugging, new consolidation) will be assigned a 3 (possible)

viii. **Date of last dose** must be clearly stated on the form

ix. **Initials** of person completing each row should be added

Multi-Dose Trial of Gene Therapy in Cystic Fibrosis Patients
GRADING TABLE FOR ADVERSE EVENTS

	Adverse Event	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 or Potentially Life Threatening	Ref
RESPIRATORY	**Lung Function**	Fall of 10-15% from baseline FEV_1	Fall of >15% - 25% from baseline FEV_1	Fall of >25% from baseline FEV_1		2
	Oxygen Saturation	Fall in SpO_2 to <95%	Fall in SpO_2 to <92%	Fall in SpO_2 to <88% or requiring supplemental O_2	Any SpO_2 requiring assisted ventilation	2
	Isolated Cough	Increased cough not requiring any new prescribed intervention	Increased cough and requiring new prescribed medical treatment	Severe, uncontrollable cough with limitation in ADLs and requiring intravenous antibiotics and/or hospital admission		3/2
	Wheeze	Mild symptoms; new intervention not indicated	Symptomatic & limiting instrumental ADL; medical intervention indicated	Limiting self-caring ADLs; oxygen saturation decreased	Life-threatening respiratory/ haemodynamic compromise; Intubation or urgent intervention indicated	3
	Shortness of Breath	Mild shortness of breath compared to normal state	Shortness of breath with minimal exertion (compared to normal) limiting instrumental ADLs	Shortness of breath at rest limiting self-care ADLs	Shortness of breath requiring urgent medical intervention	3/2
	Bronchospasm post induced sputum	Fall of 10-15% from baseline FEV_1	Fall of >15% - 25% from baseline FEV_1	Fall of >25% from baseline FEV_1		2
	Isolated chest pain	Mild pain (1-3/10); Not requiring treatment	Transient & moderate severity (4-7/10); Simple treatment required	Severe pain (8-10/10); Limiting self care or requiring hospital admission		2/ 3

CFGT/ Adverse Events Grading Table (Version 4) – 01 July 2013

	Adverse Event	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 or Potentially Life Threatening	Ref
	General Deterioration in Respiratory Status* **Defined by 2 or more of the following (severity recorded by most severe individual parameter)**	**FEV_1:** Fall of 10-15% from baseline FEV_1 **SpO_2:** Fall in SpO_2 to <95% **Cough:** Increased cough not requiring any new prescribed intervention **Breathlessness:** Mild shortness of breath compared to normal state **Chest Pain:** Mild pain (1-3/10); Not requiring treatment **Sputum Production:** Not requiring treatment	Fall of >15% - 25% from baseline FEV_1 Fall in SpO_2 to <92% Increased cough and requiring new prescribed medical treatment Shortness of breath with minimal exertion (compared to normal) limiting instrumental ADLs Transient & moderate severity (4-7/10); Simple treatment required Requiring oral antibiotic treatment	Fall of >25% from baseline FEV_1 Fall in SpO_2 to <88% or requiring supplemental O_2 Severe, uncontrollable cough with limitation in ADLs and requiring intravenous antibiotics and/or hospital admission Severe, uncontrollable cough with limitation in ADLs and requiring intravenous antibiotics and/or hospital admission Severe pain (8-10/10); Limiting self care or requiring hospital admission Requiring intravenous antibiotics and/or hospital admission	Any SpO_2 requiring assisted ventilation Shortness of breath requiring urgent medical intervention	2

Appendix A
Multidose Trial Adverse Events Grading Table

	Adverse Event	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 or Potentially Life Threatening	Ref
CONSTITUTIONAL SYMPTOMS	**Fever** (°C)	37.7 – 38.5	38.6 – 39.5	39.6 – 40.5	>40.0 for >24-hours	1/3
	Fatigue	Fatigue relived by rest; No decrease in ADLs	Fatigue not relieved by rest; Reduction in activity or ADLs by 25-50%	Fatigue not relieved by rest; Self-caring ADLs limited; unable to work; activity reduced >50%		1/3
	Myalgia	Mild pain (1-3/10); No interference with activity levels	Moderate pain (4-7/10); Some limitation with instrumental ADLs	Significant pain (8-10/10); Limitation to self-caring ADLs		4
	Headache	Transient, mild severity (1-3/10); No treatment required	Moderate severity (4-7/10) or lasting >24hrs; Limiting instrumental ADLs; Simple treatment required	Severe pain (8-10/10); Limiting self-caring ADLs; Requiring narcotic therapy		1/3/4
	Flu-like Symptoms^ **- defined by 2 or more of the following (severity recorded by most severe individual parameter)**	Mild flu-like symptoms no treatment required/no reduction in ADLs **Fever:** 37.7 – 38.5 **Fatigue:** Fatigue relived by rest; **Myalgia:** Mild pain (1-3/10) **Headache:** Transient, mild severity	Moderate symptoms; limiting instrumental ADLs; Simple treatment required 38.6 – 39.5 Fatigue not relieved by rest Moderate pain (4-7/10) Moderate severity (4-7/10) or	Severe symptoms; limiting self-care ADLs; Hospital Assessment & treatment required 39.6-40.5 Fatigue not relieved by rest Significant pain (8-10/10) Severe pain (8-10/10)		3

CFGT/ Adverse Events Grading Table (Version 4) – 01 July 2013

	Adverse Event	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 or Potentially Life Threatening	Ref
		(1-3/10)	lasting >24hrs			
	Allergic reaction	Transient flushing or rash; Pruritis. No interruption to intervention and no treatment required.	Localised Urticaria; Intervention interruption indicated; Responds promptly to symptomatic treatment	Generalised urticaria or angioedema ; Prolonged (i.e. not rapidly responsive to symptomatic medication and/or brief interruption of infusion)	Anaphylaxis; Recurrence of symptoms following initial improvement; Hospitalisation indicated	1/3
	Throat irritation	Mild discomfort or irritation; No medical intervention required	Moderate discomfort or irritation; Simple oral medication or therapy required.	Severe discomfort or irritation; reduced oral intake. Requiring medical intervention/IV therapy.		2/3

"New" Microbacterial Growths – Any organism that has NOT been cultured within the last 2 years are classified as "new" and will be recorded as an adverse event; those previously identified within the past 18 months are open to local clinical discretion/judgment, with the **only exceptions being the exclusion organisms** – *MRSA, B. cepacia complex,* and *M.abcessus* (any culture, anytime = AE) *(Ref 2)*

Appendix A
Multidose Trial Adverse Events Grading Table

	Adverse Event	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 or Potentially Life Threatening	Ref
GASTROINTESTINAL/RENAL	**Constipation**	Occasional or intermittent symptoms; occasional use of stool softeners, laxatives, dietary modification, or enema	Persistent symptoms with regular use of laxatives or enemas; limiting instrumental ADL	Severe constipation requiring high-dose laxatives and/or manual evacuation indicated; limiting self care ADL	Intestinal obstruction (inc. DIOS) with life-threatening consequences and/or urgent intervention indicated	3/1/2
	Diarrhoea	Transient; Increase of <4 loose stools/day over baseline	Increase of 5-7 loose stools/day over baseline	>7 loose stools/day; Incontinence; Limiting self-care ADLs; Orthostatic hypotension or requiring IV fluids	Hypotensive shock or hospitalisation for IV fluid replacement required	3/1
	Vomiting	Transient emeis 1 - 2 episodes (separated by 5mins) in 24 hrs; No interference with normal activity	Occasional/moderate vomiting; 3 - 5 episodes (separated by 5mins) in 24 hrs; Some interference with instrumental ADLs	>6 episodes (separated by 5mins) in 24 hrs; Orthostatic hypotension or IV fluids required; Significantly prevents self-caring ADLs	Hypotensive shock or hospitalisation for IV fluid replacement required	3/4
	Weight Loss (Unintentional)	2 – 3% from baseline	3 – 5% from baseline	>5% from baseline		3
	Abdominal pain	Mild pain (1-3/10); No medical intervention required	Moderate pain (4-7/10); Requiring medical therapy; Limitation in instrumental ADLs	Severe pain (8-10/10); Requiring narcotic therapy; Limitations in self-caring ADLs		2/3
	Urinalysis: Proteinuria	1+	2-3+	4+	Nephrotic Syndrome	1
	Urinalysis: Haematuria	Microscopic only (=dipstick positive)	Gross, no clots	Gross with clots	Urinary obstruction or blood transfusion	1

CFGT/ Adverse Events Grading Table (Version 4) – 01 July 2013

	Adverse Event	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)	Grade 4 or Potentially Life Threatening	Ref
					required	
HAEMATOLOGY/BIOCHEMISTRY	**White Cell Count ($x10^9$/L)**	Rise of 5 – 10 above baseline	Rise of 10 – 20.0 above baseline	Rise of >20.1 above baseline		2/4
	C-Reactive Protein (mg/L)	Rise of 20 – 49 above baseline	Rise of 50 – 99 above baseline	Rise of >100 above baseline		2
	Rise in ESR (mm/hr)	ESR 22 – 30	ESR 31 – 50	ESR >51		2
	Serum Bilirubin	1.5 – 1.9 x ULN	2.0 – 3.0 x ULN	3.1 – 5.0 x ULN	>5.0 x ULN	1
	Serum Creatinine	1.1 – 1.5 x ULN	1.6 – 3.0 x ULN	3.1 – 6.0 x ULN	>6.0 x ULN or required dialysis	1
	Serum Transaminase (AST/ALT)	1.5 – 2.5 x ULN	2.6 – 5 x ULN	5.1 – 10 x ULN	>10 x ULN	1
	Serum Amylase (U/L) **(a) Absolute Value** **(b) Increase from Baseline**	(a) 110 – 150 or (b) Increase by 50 – 75	(a) 151 – 200 or (b) Increase by 76 – 100	(a) >200 or (b) Increase by >100	-	2

References:

(1) WHO Toxicity Grading Scale for Determining the Severity of Adverse Events, 2003
(2) As defined locally
(3) Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0, US Department of Health and Human Sciences, Published: May 2009 (v4.03: June 2010)
(4) Guidance for Industry – Toxic Grading Scale for Healthy Adult & Adolescent Volunteers Enrolled in Preventative Vaccine Clinical Trails, US Dept of Health & Human Sciences, Food & Drug Administration, September 2007

Appendix B – 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	$CaCl_2 \cdot 2H_20$	2.25	0.33
Magnesium Chloride Hexahydrate	$MgCl_2 \cdot 6H_20$	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 (ZC):

Compound		Concentration	
		mM/L	g/L
Sodium Gluconate	$C_6H_{11}NaO_7$	135	29.43
Calcium Gluconate Anhydrous	$C_{12}H_{22}CaO_{14}$	2.2	0.95
Monobasic Potassium Phosphate	K_2HPO_4 USP	2.4	0.42
Dibasic Potassium Phosphate Anhydrous	KH_2PO_4	0.4	0.24
Magnesium Sulphate $7H_20$	$MgSO_4 \cdot 7H_2O$	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 (ZCA)

Amiloride 0.03 mg/ml (0.1 mM) is dissolved in a ZC Solution (Solution 2).

This solution is used for 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 the 'base' solution and agitated.

Isoprenaline must be added just prior to use, owing to an expiry time of 2 hours from the time dissolved.

(i) ZERO CHLORIDE + AMILORIDE (0.1 mM) + ISOPRENALINE (0.01 mM) SOLUTION (ZCAI)

Isoprenaline dissolved in ZCA solution. This solution is used for measurements of nasal PD.

(ii) ZERO CHLORIDE + ISOPRENALINE (0.01 mM) SOLUTION (ZCAI)

Isoprenaline dissolved in ZC solution. This solution is used for measurements of lower airway PD. It does not contain amiloride.