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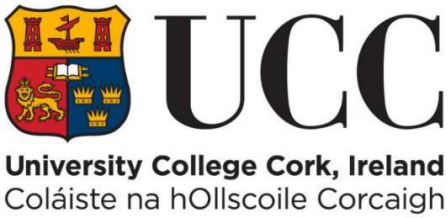

Clinical outcomes of real-world Kalydeco (ivacaftor); CORK study – understanding the implications of CFTR modulation

Thesis presented by

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Abbreviations

ABC	Adenosine-5'-triphosphate binding cassette
ABPA	Allergic Bronchopulmonary Aspergillosis
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine Triphosphate
ATS	American Thoracic Society
BAL	Broncho-alveolar Lavage
BD	Twice Daily
BMI	Body Mass Index
C DIOS	Complete Distal intestinal obstruction syndrome
cAMP	Cyclic Adenosine Monophosphate
CF	Cystic Fibrosis
CFQ-R	Cystic Fibrosis Questionnaire Revised
CFRD	Cystic Fibrosis Related Diabetes
CFTR	Cystic Fibrosis Conductance Transmembrane Regulator
Cl^-	Chloride
COPD	Chronic Obstructive Pulmonary Disease
CRP	C Reactive Protein
CT	Computed Tomography

CYP3A	Cytochrome P450 3A
DIOS	Distal intestinal obstruction syndrome
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbant Assay
EMA	European Medicines Agency
ERS	European Respiratory Society
FC	Faecal Calprotectin
FDA	Food and Drug Administration
FE-1	Faecal Elastase 1
FEV_1	Forced Expiratory Volume in 1 second
FIS	Forskolin induced swelling
FL	Faecal lactoferrin
FRT	Fischer Rat Thyroid Cells
GGT	Gamma glutyrl transferase
GI	Gastrointestinal
GIT	Gastrointestinal tract
GORD	Gastro-oesophageal reflux disease
HbA1c	Haemoglobin A1c
HBE	Human Bronchial Epithelial
HDL	High Density Lipoprotein
I DIOS	Incomplete/Impending Distal intestinal obstruction syndrome

ICS	Inhaled Corticosteroid
IFG	Impaired Fasting Glucose
IFN	Interferon
IGT	Impaired Glucose Tolerance
IL	Interleukin
IPF	Idiopathic Pulmonary Fibrosis
IRT	Immunoreactive Trypsinogen
IV	Intravenous
Kg	Kilogram
LDL	Low Density lipoprotein
MCID	Minimal Clinically Important Difference
Mg	Milligrams
MI	Meconium Ileus
MRI	Magnetic Resonance Imaging
MRSA	Methicillin Resistant Staphylococcus aureus
MSD 1	Membrane Spanning Domain 1
MSD 2	Membrane Spanning Domain 2
MSD	Mesoscale Discovery
MSv	Mili-severts
NBD	Nucleotide Binding Domain
NHS	National Health Service
NK	Negative Control

NSAIDs	Non-Steroidal anti-inflammatory drugs
OD	Once Daily
OGTT	Oral Glucose Tolerance Test
PA	Pseudomonas aeruginosa
PK	Positive Control
PKA	Protein Kinase A
PI	Pancreatic Insufficiency
PRO	Patient Reported Outcome
PS	Pancreatic sufficient
PTC	Premature Termination Codon
PWCF	Patients with Cystic Fibrosis
rRNA	Ribosomal ribonucleic acid
TNF α	Tumour Necrosis Factor α
US	United States

Declaration

I declare that this thesis represents my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Nicola Ronan

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- UCC School of Medicine New Horizons Research Conference Best Poster Presentation December 2014 for Clinical Outcomes of Real-World Kalydeco (CORK) Study.
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- Finalist in UCC "Science for all outreach" competition which aims to explain research to a lay audience March 2016 for A breath of fresh air: what happens when doors are opened in cystic fibrosis.

Chapter 1

Introduction

Introduction

Cystic Fibrosis (CF) represents one of the success stories of modern medicine with sustained incremental increases in the survival from one of childhood death to one of adult survival into the middle decades over the last thirty years. Improving survival has focused on multidisciplinary management centred on treating the consequences of this genetic disease. CF is a multisystem disorder due to mutations in the gene, which codes for cystic fibrosis conductance regulator (CFTR) protein. The *CFTR* gene resideson the long arm of chromosome 7 at position q31.2 and codes for the CFTR protein which functions primarily as an ion channel which transports chloride and bicarbonate across epithelial cell membranes in multiple organs including the lung, gastrointestinal tract (GIT), pancreas, liver and sweat glands. Whilst, respiratory manifestations dominate much of CF care, there are prominent additional multi-organ manifestations and comorbidities While the autosomal recessive pattern of inheritance of CF has been recognised since the 1940s (1), it was the identification of the gene that encodes for CF in 1989 that represents the breakthrough (2) in understanding pathogenesis and developing new treatment modalities. While historically the treatment of CF focused on the consequences of CFTR dysfunction the recent advent of CFTR modulators with the potential to enhance CFTR function represents an opportunity to potentially reverse or delay the development of some of the comorbidities associated with CF.

The horizon of therapeutics for cystic fibrosis (CF) has altered dramatically over the last number of decades, with significant successes leading to a greater than doubling of anticipated life expectancy, in this period of time (3). This success has been largely

attributed to better multidisciplinary management centred on treating the consequences of CFTR dysfunction, including nutritional care, improved anti-microbial and anti-inflammatory agents and improved physiotherapy techniques for the pulmonary component of the disease (4). The CF therapeutic pipeline has, however, more recently undergone a paradigm shift by focussing on targeting the basic genetic and/or protein defects, which lead to the clinical manifestations of disease. This approach offers significant hope for the sufferers of the condition as it holds the potential to ultimately alter disease progression or perhaps even development.

CFTR Protein

The CFTR protein is a 1480 amino acid membrane bound glycoprotein, which is a member of the ATP-binding cassette (ABC) transporters (Figure 1.1). It is comprised of five domains; two transmembrane domains, which form the channel pore, two nucleotide binding domains (NBD), which bind and hydrolyse ATP and a regulator domain which contains multiple protein kinase A (PKA) phosphorylation sites (2). In order for the channel to open – as a result facilitating chloride transport across the epithelial cell membrane - it is necessary for protein kinase A to phosphorylate the regulatory domain followed by ATP binding and hydrolysis by the two NBD's (5). Todate over 1900 disease causing mutations have been identified, giving rise to a spectrum of disease severity (6).

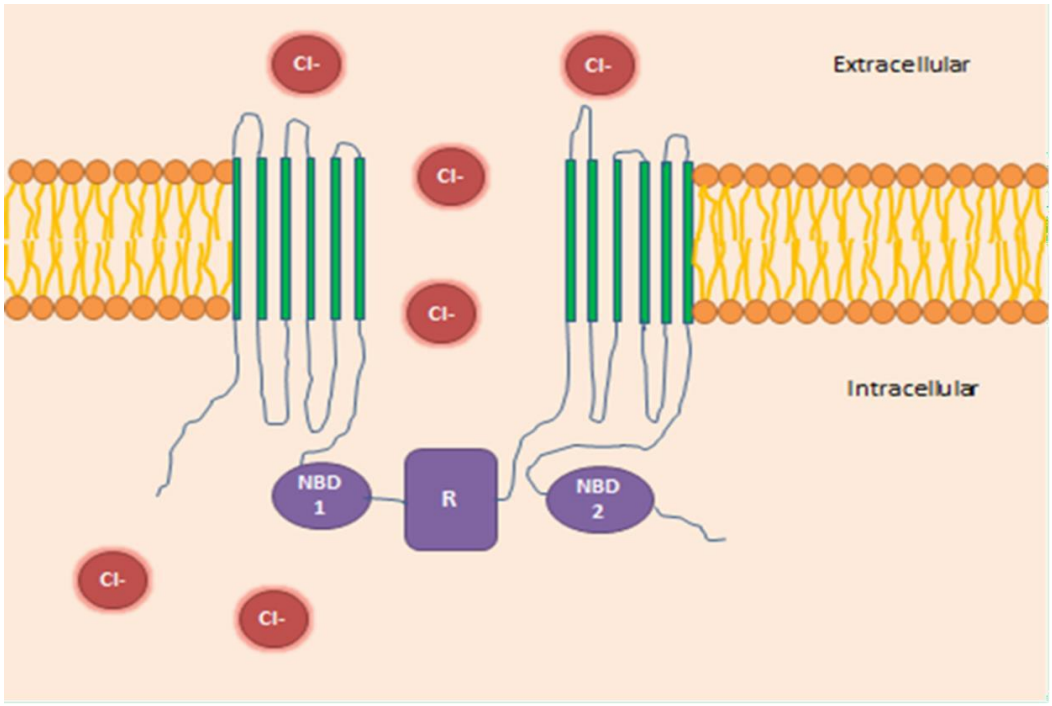


Figure 1.1 Cystic fibrosis transmembrane conductance regulatory structure. Cl-, chloride ion; NBD 1, nucleotide-binding domain 1; NBD2, nucleotide binding domain 2; R, regulatory domain.

Normal	i	ii	iii	iv	v	vi
CFTR Defect:	No Functional CFTR Protein	CFTR Trafficking Defect	Defective Channel Regulation	Decreased Channel Conductance	Reduced Synthesis of CFTR	Decreased CFTR Stability
Type of Mutations:	Nonsense; Frameshift; Canonical Splice	Missense; Amino acid Deletion	Missense; Amino acid Change	Missense; Amino acid Change	Splicing defect; Missense	Missense; Amino acid Change
Potential Therapy:	Ataluren Gene Therapy	Lumacaftor Ivacaftor / vx 661 Ivacaftor/ Gene Therapy	Ivacaftor Gene Therapy	Ivacaftor Gene Therapy	Gene Therapy	Gene Therapy

Figure 1.2 CFTR protein mutations and potential therapeutic strategies. CFTR, cystic fibrosis transmembrane conductance regulator.

Approximately 80% of these mutations can be broadly divided into 6 mutation classes (Figure 1.2), which can permit a better insight into the functional consequences of individual mutations and additionally present possible therapeutic targets (7).

CFTR mutations may be classified as follows. **Class I** mutations typically result in a quantitative defect in CFTR protein at the cell surface by preventing correct full length CFTR protein production and thus result in the production of a truncated protein which is non-functional. This can occur due to nonsense, frameshift or mRNA splicing defects. Collectively they account for approximately 10% of *CFTR* mutations worldwide and the most frequently occurring mutation in this class is G542X (8). **Class II** mutations results in a quantitative defect in protein, by producing a misfolded or immature protein which undergoes intracellular degradation and fails to reach the cell surface. Additionally, the limited amount of CFTR that reaches the cell surface opens less frequently than normal CFTR. The classic example of such a mutation is F508del, which has an estimated prevalence of 70-80% worldwide (6). By contrast classes III and IV result in a qualitative defect in CFTR with often adequate quantity of correctly localisedCFTR but a limitation in ion conductance through the channel (9, 10). **Class III** mutations are characterised by defective protein, which does not respond to cAMP stimulation, with the result that the chloride channel opens less frequently than normal and hence has a gating defect. G551D is the most common of the mutations within this class occurring in approximately 5% of patients' (8) worldwide but exhibitingsignificant geographic heterogeneity (11). **Class IV** mutations- most notably R117H - results in presence of correctly localised CFTR protein at the cell surface, but which is

narrowed and results in conductance defect. **Class V** mutations result in a reduced quantity of functional CFTR at the cell surface. **Class VI** mutations lead to increased turnover of functional CFTR at the cell surface.

CFTR Modulation – The concept and therapeutic application

The recognition that many different classes of mutations exist is notable when attempting to restore functional CFTR to the cell surface. A number of potential strategies exist for CFTR modulation with a restoration of function, including gene therapy, read-through agents, CFTR correctors and CFTR potentiators (Figure 1.3). In the first instance, modulation of CFTR can occur through targeting the genetic defect directly (12, 13). An example of this approach is genetic manipulation leading to restoration of 'normal *CFTR*' gene structure into target tissues. This approach is the cornerstone to some novel therapeutic approaches currently being investigated including gene therapy, which may provide a potential universal approach to treatment across all mutation classes. The second major target of CFTR modulation is to directly target the aberrant protein product, which is produced following translation of the mutated *CFTR* gene. Read-through agents enable ribosomal read-through of prematurestop in CFTR mRNA but not normal stop codons and are potentially useful for treating class I mutations. CFTR correctors aim to correct CFTR misfolding as occurs in class II mutations and are potentially useful in combination with potentiators for treating ClassII mutations. CFTR potentiators enhance CFTR mediated chloride transport by increasing channel opening and thereby increasing chloride transport and are potentially useful for treating class II, III and IV mutations.

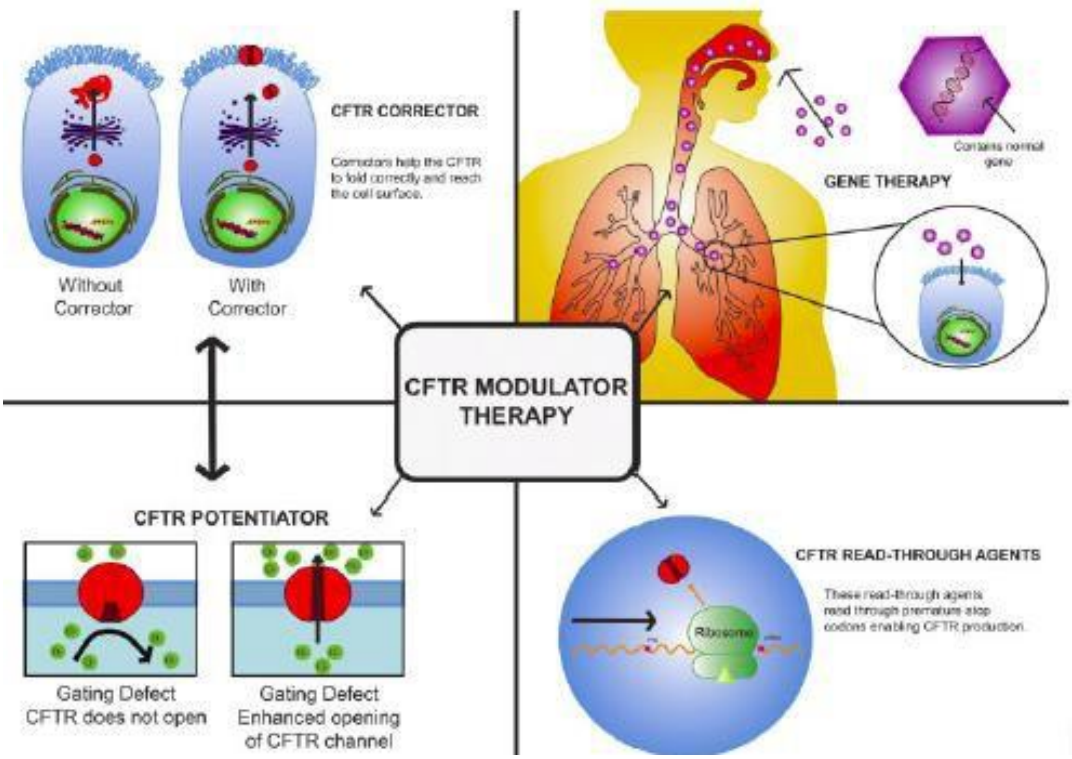


Figure 1.3 CFTR modulation the concept.

Targeting the protein directly may be more complex as it requires potentially differing approaches depending on mutation class. Furthermore, as many mutations do not fit entirely into one class and have the hallmark deficits of more than one class, combinations of therapy may be necessary to adequately restore functional CFTR protein to the cell surface. Currently all these potential therapeutic areas are being explored, however, at different stages of clinical utility. With the early success and relatively widespread clinical use of CFTR potentiation, for certain mutations, it is a useful start point in understanding CFTR modulation.

CFTR Potentiation

Early *in vitro* work suggested that using compounds including sulfonamide, phenylglycine, pyrrolo[2,3-b]pryazines and benzothiphene may be effective (14-16), however, it was the discovery of VX-770 (Ivacaftor), which represented the first compound to be proposed as a suitable medication for *in vivo* application. Ivacaftor was identified through a high-throughput screening process of over 228,000 compounds, and was modified using several rounds of analog synthesis to derive a compound with selectivity for CFTR and an attractive preclinical pharmacokinetic profile (17, 18).

Evidence that Ivacaftor enhances CFTR function is provided from a number of in vitro studies. Yu et al have demonstrated that addition of Ivacaftor to G551D Fischer Rat Thyroid (FRT) cells increased channel open probability from 5% of normal CFTR to levels equivalent to 30% of normal CFTR. Similarly, a 55.3-fold increase in chloride transport in G551D FRT cells was observed after addition of Ivacaftor. In G551D/F508del Human Bronchial Epithelial Cells (HBE) Ivacaftor has been shown to increase airway surface liquid to levels equivalent to approximately half of the level seen in non CF cells and to enhance ciliary beat frequency to near normal (17). Taken together these results suggested a strong *in vitro* response of G551D-CFTR to ivacaftor and formed the cornerstone of subsequent human studies.

Given that both phosphorylation and ATP binding are required for CFTR channel function (5, 9) it has been postulated that ivacaftor may affect one or both of these processes. In vitro analysis has demonstrated that ivacaftor

enhances CFTR channel function in a phosphorylation dependent but ATP independent manner, on the basis of the observation that addition of Ivacaftor to non-phosphorylated wild type CFTR did not increase ion flux (19). However, when added to pre-phosphorylated CFTR Ivacaftor produced a 2-fold increase in ion flux. Similarly, no baseline channel activity was observed in pre-phosphorylated wild type CFTR in the absence of ATP; however, addition of ivacaftor resulted in increased ion flux in the absence of ATP thus suggesting that Ivacaftor manages to bypass the need for ATP.

Human Studies

In a two-part phase 2 randomised double-blind placebo-controlled trial, ivacaftor, an orally bioavailable compound was studied at a series of doses in 39 adults with CF who carried at least one copy of the G551D mutation. Safety of short-term ivacaftor therapy was confirmed, and a significant within subject change in nasal potential difference was seen in the higher ivacaftor dosing groups. Additionally, a significant dose dependent reduction in sweat chloride was noted up to the 150mg dosage with a reduction in the mean sweat chloride level to below the threshold value for diagnosisin CF in the 150mg dosage group. Finally, a significant within patient increase in FEV_1 was noted in all dosing groups over 75mg twice daily.

These encouraging results led to two phase III studies; ENVISION and STRIVE (20, 21). STRIVE was a 48 week randomised, double blind, placebo controlled phase III trial conducted in 166 patients with CF aged 12 and older with at least one copy of the G551D mutation and an FEV_1 of 40-90% predicted (20). ENVISION was a 48 week randomised, double blind, placebo-controlled phase III trial conducted in 52 children

aged 6-11 with at least one copy of the G551D mutation and a baseline FEV_1 of 40 - 105% predicted (21). In each study, marked reductions were noted in sweat chloride, which was utilised as a biomarker of CFTR effect (-48.7mmol/L, *p<0.001* STRIVE, - 55.5mmol/L, *p<0.001* ENVISION). This striking change reduced mean sweat chloride levels to below the diagnostic threshold for CF, emphasising potentially profound effects on CFTR protein function. Spirometric responses were equally impressive with absolute increases in FEV_1 of 10.4% (STRIVE) and 10.7% (ENVISION) noted. Ivacaftor therapy also produced a significant 55% reduction in pulmonary exacerbations in STRIVE; with the pulmonary exacerbation rate being too low to detect a signal in ENVISION. Clinically significant improvements in the respiratory component of thecystic fibrosis questionnaire-revised (CFQ-R) were observed in STRIVE and ENVISION. Further systemic effects were noted by a significant increase in weight and BMI z- scores.

PERSIST the open label extension study of 144 adolescents/adults (≥ 12 years) and 48 children (age 6- 11 years) with the G551D mutation who previously commenced Ivacaftor as part of STRIVE and ENVISION (22) has demonstrated sustained improvement in lung function, weight and pulmonary exacerbations for up to 144 weeks of Ivacaftor therapy. In the patient's cohort treated with ivacaftor in STRIVE and ENVISION mean absolute increase in FEV_1 was 9.4% and 10.3% after 144 weeks of treatment respectively. In the cohorts who were initially treated with placebo in STRIVE and ENVISION a significant mean absolute increase in FEV_1 of 9.4% and 10.5% respectively was observed after 48 weeks of Ivacaftor. The reduction in pulmonary

exacerbations reported in STRIVE was maintained in PERSIST. At the end of 144 weeks of PERSIST, approximately 30% of those who received ivacaftor in STRIVE were still free from a pulmonary exacerbation.

Following approval of ivacaftor the 'real-world' GOAL study followed 151 CF patients carrying the G551D mutation who were prescribed ivacaftor in the United States. They reported a 6.7% mean improvement in FEV_1, a 2.5 Kg mean increase in weight, a 7.4 point mean increase in CFQ-R respiratory domain and a 53.8 mmol/l mean reduction in sweat chloride after 6 months of treatment, findings which closely mimicked those seen in clinical trials (23). There was a 16.3% decrease in total hospitalisations in the 6 months post treatment with ivacaftor compared to the matched 6-month period in the year pre-treatment with Ivacaftor.

Additionally, there now exists efficacy data for patients carrying the G551D mutation who would have been excluded from the original clinical trials. A multi-centre study of 21 patients with the G551D mutation and severe CF lung disease ($FEV_1 <$ 40% predicted) demonstrated a 16.7% relative improvement in lung function, 1.8 Kg weight gain and a significant reduction in hospitalisation and intravenous antibiotic requirements post treatment with ivacaftor (24). A safety study in children aged 2-5 years has also been completed and recently presented. The KIWI study assessed the safety, pharmacodynamics, and pharmacokinetics of ivacaftor in 34 young CF patients who carried a mutation believed to be responsive to ivacaftor (32 patients with G551D, 2 patients with S549N) (25). In this young age group, efficacy was again demonstrated by significant effects on sweat chloride and a significant increase in weight for age z-

scores. Intriguingly, this study also reported on serial faecal elastase measurements as a surrogate for exocrine pancreatic function. The authors noted an increase in faecal elastase measurements in 16/26 children who had profound exocrine pancreatic dysfunction at baseline. Although the results did not reach significance, this early signal may suggest that some CFTR related disease might be reversible in subjects who are treated at a young age.

More recently the ARRIVAL study has demonstrated the safety and benefit of ivacaftor in infants aged 4-12 months with at least one gating mutation, with a 166 μg/g mean improvement in FE-1, and -474.5 ng/ml reduction in immunoreactive trypsinogen level being reported after 24 weeks of treatment (26).

Adverse events and drug interactions

In pooled phase 2b/3 clinical trials the most frequently observed side effects observed in patients were: headache (17%), upper respiratory infection (16%), nasal congestion (16%), rash (10%), rhinitis (6%), dizziness (5%), and arthralgia (5%) (20, 27). Elevation of hepatic transaminases has also been noted in patients treated with ivacaftor; these were not significantly different to the groups treated with placebo (20, 21). While the mean liver transaminase levels were within normal limits at all visits up to week 96 of PERSIST (22), increases of liver transaminases to more than 5 times the upper limit of normal or resulting in interruption of Ivacaftor treatment was observed in nine patients. In patients aged 2-5 years in the KIWI study, a significant rise in transaminases(>8 times the upper limit of normal) was noted in 14.7% of the patients, which returned to baseline following drug interruption (25). All of these patients had been noted to have elevated

liver function tests at baseline. We await further data on potential mechanisms of this adverse event. Monitoring of transaminase levels is currently recommended on a 3-monthly basis during the first year of treatment and then annually. In PERSIST discontinuation rates due to adverse events were low (1% in the adult cohort, and 1% in the child cohort) (22). There were two deaths (1%) reported in patients from the STRIVE cohort, which were felt to be unrelated and unlikely to be related to Ivacaftor treatment.

Ivacaftor is metabolised by the cytochrome P450 pathway (CYP) via the CYP3A isoenzyme (28, 29). As numerous drugs and foods can induce or inhibit various Cytochrome P450 isoenzymes there is considerable potential for drug-drug and drug-food interactions, including many medications with use in CF (30). There are numerous drug and food interactions which may specifically increase or decrease ivacaftor exposure, including antifungals, antibiotics, foods (Seville Oranges and grapefruit), anti-epileptics and herbal remedies (31).

CFTR Potentiators in other mutations

Early clinical studies predominantly investigated patients with the G551D mutation as this mutation represents the most common of the Class III mutations. *In vitro* data has documented efficacy of ivacaftor across a range of other mutations within this class. Yu et al.demonstrated the ability of Ivacaftor to increase channel open probability and chloride transport in FRT cells expressing gating mutations - including G551D, G551S, G1349D, G1244E, G970R, G178R, S549N, S549R, S1251N, and S1255P (32). The effect of

ivacaftor on 54 *CFTR* mutations was evaluated by Van Goor et al (33). Ivacaftor was shown to potentiate 34 of the 54 *CFTR* mutations, as reflected by a 1.5 – 55.3-fold increase in chloride transport post-ivacaftor; corresponding to levels equivalent to 2.1% - 200.7% of normal CFTR function. Responsive mutations included those in other classes of mutations for example Class IV as noted by the significant response noted in R117H and D1152H mutations and some Class II mutations. These results suggest that CFTR potentiation therapy may have clinical applications outside of G551D.

KONNNECTION was a phase III randomised double blinded placebo controlled trial, which evaluated ivacaftor in Class III mutations other than G551D, including; G178R, S549N, S549R, G551S, G970R, G1244E, S1251N, S1255P and G1349D (34). After 24 weeks of therapy, mean change in absolute FEV_1 was 13.5% and similar improvements in sweat chloride and weight to those seen in the G551D studies were noted. An interesting finding revealed a lack of sweat chloride or clinical response in the 4 patients who had the G970R mutation. This finding was unexpected due to a strong indication from *in vitro* data that this mutation would be responsive (33). Interestingly, more recent organoid work demonstrated that G970R organoids did not respond to ivacaftor monotherapy (35). Further organoid RNA analysis showed that 3 different splice variants were created by G970R *CFTR* and ultimately demonstrated that cryptic splicing is an uncommon reason of mutation misclassification in cell lines (35). Case reports have also supported the use of ivacaftor in a patient with severe lung disease carrying the S549N mutation (36).

The efficacy of ivacaftor in Class IV mutations has been examined in clinical trials

including the KONDUCT and KONTINUE studies, and the recently presented N-of-1 studies (37, 38). KONDUCT and KONTINUE evaluated the effects of Ivacaftor in patients carrying the R117H mutation, which is notable for a heterogeneous phenotype. KONDUCT was a 24 week phase double blinded placebo controlled trial involved 69 patients ≥ 6 years with at least one copy of the R117H mutation and a baseline FEV_1 ≥ 40% predicted (38). This study did not reach its primary endpoint of change in FEV_1 % predicted at 24 weeks (2.1% absolute change, *p=0.2*). A pre-specified subgroup analysis based on age revealed that adult patients had a significant increase in lung function(5% absolute change, *p = 0.01*), but lung function significantly declined in those patients aged 6-11 years. A significant sweat chloride reduction was seen for the entire cohort (-24.0 mmol/L, *p<0.0001*), and a clinically significant increase in the CFQ-RRespiratory Domain scores (+8.4, *p<0.01*). In the interim 12-week analysis of the KONTINUE study - an open label extension of ivacaftor in those patients entered into KONDUCT following a wash out period, a significant increase in pulmonary function was seen for the entire cohort (5.5% absolute change, *p<0.001*). There was no significant change in nutritional status as measured by BMI, but the overall cohort tended to be well nourished at baseline with the vast majority being pancreatic sufficient.

A significant barrier to broadening the applicability of CFTR potentiation is the relative rarity of a number of mutations. We have seen promising in *vitro* responses not translating into clinically meaningful improvement (33, 34). Sample size may confound this (34). An effort to target these rarer mutations was made in the novel recently presented N-of-1 study, which evaluated the effects of ivacaftor in 21 patients with

residual function mutations in a complex cross over design involving 2 2-week treatment periods of each ivacaftor and placebo, an 8 week open label ivacaftor treatment period and intervening washout periods (39). This study suggested a clinical response in most patients across a range of outcome measures with no new safety concerns identified. We await further reports to assess whether there are factors, which may be able to correctly identify mutation specific responders by utilising this approach.

Finally, the efficacy of ivacaftor has been tested in F508del homozygote patients (40). This study was conducted primarily to gain additional safety information and to determine whether the small effects of ivacaftor on F508del-CFTR chloride secretion noted *in vitro* would have a clinical impact. As was expected, there was no significant improvement in any clinical measure, but interestingly a small but statistically significant reduction in sweat chloride was found suggesting that any F508del that is trafficked to the cell surface *in vivo* may have a resultant gating defect (40).

CFTR Correctors

In Class II mutations, adequate protein does not reach the cell surface due to folding and trafficking defects. Compounds have been identified from pre-clinical studies (14, 41-43) but to date only 2 compounds have been utilised in clinical trials.

Lumacaftor (VX-809) is a small molecule compound identified through high throughput screening. The initial *in-vitro* studies revealed VX-809 produced a 7.1 fold improvement in F508del CFTR maturation, which was accompanied by a five-fold improvement in chloride transport (44). The addition of VX-809 to HBE cells homozygous for F508del mutation resulted in an eight fold increase in CFTR maturation and a four-fold increase in chloride transport (44). This represented a rise in chloride transport from 3.4% to

13.9% of the level of transport seen in non-CF HBE. Finally, it was observed that the addition of ivacaftor increased chloride transport to approximately 25% of the level seen in non-CF HBE, suggesting that the addition of a CFTR potentiator would be necessary to produce clinically meaningful improvements for corrected mutant F508del-CFTR.

From a mechanistic viewpoint, lumacaftor (VX-809) appears to act downstream on the first transmembrane spanning domain (MSD1) (45). This results in partial correction of the folding defect associated with F508del CFTR by improving the interaction between NBD1, MSD1 and MSD2 (Figure 1.2). *In vitro* evidence suggested a potential detrimental interaction on combinations of ivacaftor with lumacaftor, with chronic ivacaftor therapy potentially increasing turnover, reducing the folding efficacy and post processing stability in post-ER compartments of Lumacaftor corrected F508del CFTR (46, 47). Vx-661 (Tezacaftor) was developed from lumacaftor in an effort to reduce drug-drug interactions with ivacaftor (48). Vx-445 (Elexacaftor) is a next generation CFTR corrector. In vitro the combination of elexacaftor-tezacaftor-ivacaftor significantly improved F508del CFTR protein processing and chloride transport (49-51).

There are several other correctors in development (50, 52).

Human studies

The initial phase II study of lumacaftor evaluated its use as monotherapy in a randomised, double-blind, placebo controlled trial evaluated in 89 patients, who were homozygous for the F508del mutation (40). Patients were enrolled into 2 separate cohorts and randomised to receive VX-809 25mg, 50mg, 100mg or 200mg once daily or placebo for 28 days. A dose dependent reduction in sweat chloride was observed within

7 days and was significant at 28 days for the 100mg (-6.13 mmol/l, P <0.05) and 200mg groups (-8.21 mmol/l, P <0.01). This study confirmed the short-term safety of lumacaftor but did not result in any significant changes in clinically relevant outcome measures such as spirometry or quality of life measures.

A further phase II trial evaluated lumacaftor monotherapy for 14 -28 days - depending on the cohort to which the patient was assigned - followed by a period of combination therapy with ivacaftor in a complex study with multiple dosing regimens in CF patients with either an F508del homozygote or heterozygote genotype (53). Lower dose lumacaftor monotherapy (200mg) in a homozygote population resulted in a small but significant reduction in sweat chloride, which was augmented further during the period of combination therapy with ivacaftor 250mg BD (-9.1mmol/L, *p<0.001*). No significant changes were noted in lung function compared to the placebo group. Further cohorts - included 27 heterozygote patients and 97 homozygotes treated with lumacaftor monotherapy for 28 days followed by later addition of ivacaftor. Interestingly a dose dependent downward trend in lung function was observed in the homozygotes during the period of lumacaftor monotherapy. During the period of combination therapy, a significant increase in FEV_1 compared to placebo was observed in the Lumacaftor400mg bd (6.1% mean increase FEV_1 *p=0.004*) and 600mg od (6.2% mean increase FEV_1, *p<0.001*) groups. No significant change in FEV_1 was observed in the F508del heterozygotes either while on lumacaftor monotherapy or lumacaftor/ivacaftor combination therapy.

Results from two phase III studies; TRAFFIC and TRANSPORT involving 1,108 F508del homozygote patients older than 12 years, demonstrated benefit in this cohort (54, 55).

The two studies had identical entry criteria and evaluated the combination of ivacaftor 250mg BD with either lumacaftor 400mg BD or 600mg OD for 24 weeks (55, 56). Pooled results from both studies demonstrated a significant mean absolute increase in FEV_1% predicted in both dosing cohorts compared to placebo (2.8% *p<0.0001*, 3.3% p<0.0001 respectively). A significant increase in BMI was also noted in both dosing cohorts (0.24kg/m^2 *p=0.0004*, 0.28kg/m^2 *p<0.0001* respectively). Most notable perhapswas the significant decline in the rate of pulmonary exacerbations (rate ratio: 0.61 *p<0.0001*, 0.70 *p=0.0014* respectively). These results translated into a significant reduction in events requiring IV antibiotics (45% reduction in the 600mg dosing arm, 56% in the 400mg BD arm).

Tezacaftor (Vx-661) is a CFTR corrector with a similar mechanism of action to lumacaftor, but has a longer half-life and less potential to interact with other medications compared to Lumacaftor (57). VX-661 has been evaluated as monotherapy at doses of 10mg, 30mg, 100mg and 150mg od and in combination with Ivacaftor (150mg bd) in a phase II randomised double blinded placebo-controlled trial (57, 58). In 128 patients aged ≥ 18 years, homozygous for the F508del mutation, no sustained significant change in lung function was observed with VX-661 monotherapy. Statistically significant mean relative improvements in FEV_1 were observed in the groups treated with Ivacaftor in combination with doses of VX-661 of 100mg (FEV_1 9%, P = 0.01) and 150mg (FEV_1 7.5%, P = 0.02) after 28 days of treatment.

The phase III randomised double-blind placebo controlled EVOLVE study evaluated combination treatment with tezacaftor-ivacaftor in patients with CF aged 12 and older

homozygous for the F508del mutation (59). A 3.4% mean absolute increase in FEV_1 % predicted was observed in the tezacaftor-ivacaftor group after 24 weeks of treatment (*p <0.001*). The improvement in FEV_1 was paralleled by a 35% reduction in pulmonary exacerbations in the tezacaftor-ivacaftor group. In the tezacaftor-ivacaftor group there was a mean improvement of 5.1 points in the CFQ-R respiratory domain and a mean 10.1 mmol/l reduction in sweat chloride (59). The phase III EXPAND study assessed tezacaftor-ivacaftor in patients with CF aged 12 and older with heterozygous for the F508del mutation and one residual function mutation (60). Tezacaftor-ivacaftor combination therapy demonstrated significant improvement in FEV_1 compared to placebo and ivacaftor monotherapy respectively (60).

More recently the next generation CFTR modulators Vx-445 and Vx-659 have been evaluated (49, 61). Like tezacaftor they are small molecule correctors which improve F508del CFTR trafficking. However, they have a different mechanism of action to tezacaftor. Thus, it was postulated that triple therapy treatment with tezacaftor, ivacaftor and oneof these next generation corrects would provide additional benefit over dual therapy inpatients homozygous for the F508del mutation or with one copy of the F508del mutation and a minimal function mutation. Combination triple therapy with VX659- tezacaftor-ivacaftor demonstrated improvement in chloride transport in human bronchial epithelial cells with F508del-F508del mutation or F508del-minimal function (61). Greater improvements in chloride transport were observed with triple therapy that monotherapy or dual therapy. In the phase II efficacy component of this study a 9.7% mean absolute improvement in FEV_1 % predicted was observed in the F508del

homozygous group and a 10.2-13.3% mean absolute improvement in the F508del-minimal function group depending dose. Significant improvements were also observed in sweat chloride and respiratory domain of the CFQ-R (62). Treatment with Vx-445-tezacaftor-ivacaftor resulted in similar improvements in FEV1, reduction in sweat chloride and improvement in CFQ-R respiratory domain in both F508del homozygous and F508del-minmal function cohorts(49, 62).In phase III randomised controlled trials elexacaftor-tezacaftor-ivacaftor in patients heterozygous for the F508del mutation and a minimal function mutation resulted in a 13.8%-14.3% mean increase in FEV1, 63% reduction in pulmonary exacerbations, 20.2 mean increase in CFQ-R respiratory domain and 41.8mmol/l mean reduction in sweat chloride at 24 weeks (63). In phase III trials involving patients homozygous for the F508del mutation experienced a 10% mean increase in FEV1, 45.1 mmol/l mean reduction in sweat chloride, and a 17.4 point mean improvement in CFQ-R respiratory domain (62).

Read-through agents

As previously stated, an alternate approach to restoring sufficient CFTR protein to the cell surface is to target the genetic defect directly. Mutations that code for premature stop codons (PTC) account for approximately 5-10% of all *CFTR* mutations, with regional variation. These agents act by encouraging ribosomal read-through of the premature stop codon with resultant formation of mature CFTR.

Howard and colleagues demonstrated the ability to restore full length CFTR in cells expressing premature stop codon *CFTR* mutations, when treated with the aminoglycoside G-418. Gentamicin was observed to produce a small effect (64). Early

human studies of the use of either intravenous or nasal gentamicin, reported improvements in nasal potential difference measurements and improved detection of mature CFTR at the cell surface (65, 66). In a double-blind, placebo controlled, crossover trial, nasal administration of gentamicin resulted in a significant reduction in nasal potential difference for patients who were homozygote or heterozygote for PTC mutations but not for F508del homozygotes (67). Subsequent studies have not been universal in identifying benefit (68, 69). Newer agents have been advocated due to the potentially limiting toxicities associated with systemic aminoglycoside therapy (70, 71) Xue et al. reported on the *in vitro* efficacy of an aminoglycoside derivative whose action was further enhanced by CFTR potentiation (72). Importantly the described molecule NB124 was noted to be less cytotoxic than gentamicin and this or similar agents may offer potential as future therapy.

The read-through agent ataluren (formerly PTC124) is an oral agent, which has been tested in phase III clinical trials in CF and remains a molecule of significant interest. It is currently licensed by the European Medicines Agency (EMA) for patients aged ≥ 5 years old who have Duchenne's muscular dystrophy and a nonsense mutation and are still ambulatory. Ataluren (10mg/kg morning and midday and 20 mg/kg in the evening) was evaluated in phase III randomised double blinded placebo controlled trial in 232 patients aged ≥ 6 years with CF with class I mutations and a baseline FEV_1 of 40 –

90 % predicted (73). The change in lung function and number of pulmonary exacerbations at 48 weeks compared to placebo was not significantly different. Similarly, no significant change in sweat chloride (-1.3 mmol/l mean reduction), BMI, nasal potential difference or CFQ-R was observed in the ataluren group after 48 weeks of

treatment. However, subsequent subgroup analysis of patients not receiving inhaled tobramycin showed a 5.7% difference in relative change in FEV_1 in the ataluren group compared to placebo and reduction in pulmonary exacerbations in the ataluren treated group (Rate ratio 0.6). As a result, a further study of ataluren in patients withCF and a nonsense mutation who were not receiving chronic inhaled tobramycin is was conducted. However, this failed to demonstrate significant benefit with a disappointing 0.6% FEV_1 benefit being reported with ataluren compared to placebo

Gene Therapy

Since the discovery of the CF gene, there has been keen interest into whether it is potentially possible to restore wild-type *CFTR* DNA into human cells. This requires the development of suitable vectors to transport the DNA into host cells. Viral vectors have been explored but have been limited somewhat by activation of the hosts immune response and therefore an attenuation of response with repeated dosing (74). To address these issues, non-viral vectors have also been studied to assess their suitability for gene transfer (75). The clinical applicability of these vectors was also initially limited by induction of a host immune response. Recently The UK Gene Therapy Consortium has completed a phase III trial in CF patients utilising a modified plasmid coupled to a cationic lipid carrier vector, and results are awaited (76).

CFTR Modulation as an insight into pathophysiology

Partially correcting the CFTR defect has permitted key insights into disease process in CF. It has been established that ivacaftor therapy results in significant improvement in pulmonary function and a reduction in pulmonary exacerbations. Studies and case

reports have reported improvement in high resolution CT Thorax scoring of mucus plugging, bronchiectasis and airway wall thickness after Ivacaftor treatment (77, 78). These imaging studies provide some insight into potential reversible components of CF lung disease and may explain how even patients with severe CF lung disease may benefit from modulation therapy. It has been suggested that techniques such as Hyperpolarised Nobel Gas Magnetic Resonance Imaging may provide a more sensitive evaluation than FEV_1 of early lung disease in patients with CF (79-81). In a small study this technique was demonstrated to be responsive to changes following ivacaftor therapy revealing a 30.4% reduction in ventilation defect volume after 2 weeks of Ivacaftor and 38.6% reduction after 4 weeks (82). Similarly, lung clearance index (LCI) may be a more sensitive marker of treatment response in patients with a normal FEV_1. In a phase II double blinded cross-over placebo-controlled trial, 17 patients aged ≥6 years with the G551D mutation and an FEV_1 > 90% predicted completed LCI pre and post Ivacaftor (83). A significant 2.16 mean reduction from baseline in LCI was observed post-Ivacaftor ($P < 0.0001$) denoting a reduction in ventilation heterogeneity. These findings of reduction in ventilation heterogeneity and ventilation defects, combined with the reduction in mucous plugging and airway thickening in HRCT studies, would suggest that CFTR restoration may affect the clearance of thickened mucous *in* vivo. The findings of the GOAL study, where a subset of patients demonstrated significant improvements in peripheral and whole lung mucociliary clearance as measured by gamma-scintigraphy support this (23). These findings together may reflect the previously outlined *in vivo* responses of increasing apical surface liquid and ciliary beat frequency (17) and provide

a key translational insight into the maintenance of the lung environment.

It was suggested that these improvements witnessed post CFTR modulation would be associated with a reduction in the microbiological and inflammatory burden that is encountered in CF lungs (84, 85). Additionally, ivacaftor contains a quinolone ring, which may afford it some degree of antimicrobial activity. In severe patients, there wasno change in the respiratory pathogens detected by conventional culture pre- and post-ivacaftor (24). In a less severe patient cohort, Rowe et al demonstrated an 18.8% reduction in the number of patients with at least one positive respiratory culture of *Pseudomonas aeruginosa* in the 6 months following ivacaftor commencement compared to 6 months prior (23). Interestingly, this change was not fully reflected in the subset of patients who underwent molecular testing of their respiratory microbiome. This revealed no significant change in sputum bacterial diversity post- treatment but identified a downward trend in the combined relative abundance of traditional CF pathogens. Similarly, they did not note a change in sputum inflammatory markers including neutrophil elastase, IL-1β, IL-6 and IL-8 (23). It is challenging to balance these findings with the noted reduction in pulmonary exacerbations in both phase III and real-world studies, and this provides an area of potential research, which may ultimately provide a greater understanding of pulmonary exacerbations and microbiology.

Improvements in the respiratory tract are not limited to the lungs and case reports and a small study (N = 12) have demonstrated improvement in severity of sinus disease on CT imaging after ivacaftor therapy compared to baseline imaging (86, 87). The GOAL study also reported improved sinus disease symptoms post ivacaftor introduction as

measured by questionnaire (23). This is equally interesting given the STRIVE data reported increase upper respiratory tract symptoms and sinusitis post-Ivacaftor. Following this important aspect of airways disease will be useful.

In the pancreas CFTR dysfunction may result in disruption of pancreatic exocrine and endocrine function giving rise to pancreatic insufficiency (PI) and CF Related Diabetes (CFRD) respectively. As previously outlined, findings of the KIWI study have for the first time suggested the potential of improving exocrine pancreatic function with early modulation of CFTR (25). These findings need to be followed longitudinally to evaluate the potential stability and reproducibility of these changes and to assess whether those subjects with partially restored exocrine pancreatic function may become more susceptible to pancreatitis as is witnessed in nominally pancreatic sufficient CF patients. Recent case reports mimic these findings and raise the possibility of a restoration of pancreatic exocrine function permitting a reduction in and/or discontinuation of pancreatic enzymes in patients treated with ivacaftor (88, 89). A reduction in pancreatic enzyme requirements may be explained by the observation that intra-luminal gut pH increases following ivacaftor therapy (23) and thereby may render pancreatic enzyme supplementation more effective.

Endocrine pancreatic function may also be influenced by ivacaftor therapy. A small study of ivacaftor use in patients with CF with the G551D mutation reported on a 66- 178% improvement in OGTT after 4 weeks of treatment with Ivacaftor. A 51-346% improvement in acute insulin secretion after Intravenous glucose was observed in all except one patient who had new onset untreated diabetes (90). CF Registry of Ireland

data demonstrated a 2.26% increase in FEV_1 per annum in patients under 12, no change in FEV_1 in 12–18-year-olds and a 1.73% decline in FEV_1 per annum in patients older than 18 years in the 36 months after commencing ivacaftor (91). A 33% reduction in hospitalisation was also noted (91). US and UK CF registry data have demonstrated a lower risk of death, transplantation and hospitalisation in ivacaftor treated G551D patients with CF compared to non-ivacaftor treated patients (92). It also demonstrated CF associated complications to be less common in ivacaftor treated patients with CF with statistically significantly lower rates of CF-related diabetes, hepatobiliary disease, bone and joint complications and depression being reported (92). While GI and pulmonary complications were less frequently reported in ivacaftor treated patients it was not statistically significant (92). Interestingly *Pseudomonas aeruginosa* and *Aspergillus species* were both less commonly reported in ivacaftor treated patients. A recent Canadian CF registry study suggested a reduced annual rate of decline in lung function after ivacaftor in addition to a reduced odds of a positive *Pseudomonas aeruginosa* sputum culture after treatment (93).

Outcome measures and biomarkers of treatment response

In medicine the efficacy of a treatment or therapeutic intervention describes whether the treatment of interest produces a benefit compared to a placebo or another treatment when evaluated under optimal conditions; typically resource rich setting such as those present in a clinical trial in a highly selective patient cohort (94, 95). In contrast the effectiveness of a treatment refers to the benefit the treatment produces in a real-world setting. It is not uncommon for the effectiveness of a medication to be lower than

its efficacy due to a variety of factors. Patients with comorbidities, severe disease, mild disease, or compliance issues are often excluded from clinical trials. Thereis also greater potential for drug-drug interactions in a real-world setting. In clinical trials in CF patients with an FEV_1 < 40% predicted or an FEV_1 >90% predicted, organisms associated with accelerated decline in lung function (e.g. *Burkholderia cepacia complex*, non-tuberculous mycobacteria) are frequently excluded from clinical trials. Asa consequence, the patients included in drug studies may not be representative of the average/ typical patient who will ultimately be treated with the medication.

Clinical trials assess the efficacy of a treatment using accepted outcome measures. In order for an outcome measures to be of utility as a primary endpoint in a clinical trial it must severe as a marker of disease severity and improve reproducibly with disease improvement (96). The US FDA outlines clinical endpoints as markers of function or patients' symptoms and surrogate endpoints as laboratory tests which may serve as a biomarker of a clinical endpoint (97, 98). Clinical endpoints which are often reported in CF include Forced expiratory volume in one second (FEV_1), pulmonary exacerbation rate, and patient reported quality of life. While these are useful outcome measures, they are not without their own limitations. Thus, this thesis aims to evaluate the real- world effectiveness of ivacaftor on a variety of traditional and emerging potentially useful outcomes measures.

Sweat chloride has been suggested as a potential biomarker of CFTR modulation, based on the ivacaftor treatment response in clinical trials. Some health services are incorporating sweat chloride thresholds in to criteria for continuation of therapy (99).

However, analysis of both clinical trial data and 'real-world' data suggest that there is no significant linear relationship between the magnitude of change in sweat chloride and the improvement in FEV_1 either in absolute or relative terms (100) and that there is no minimum change in sweat chloride which is predictive of an increase in FEV_1 in patients treated with Ivacaftor (101). Re-analysis of trial data has suggested certain threshold measures at 15 days of Ivacaftor treatment which were useful in predicting patients who will have a ≥ 5 % increase in FEV_1 after 16 weeks of treatment (102). However, the negative predictive value of these thresholds may limit their clinical utility. Furthermore, the sweat chloride response in phase II clinical trials of combined correctors and potentiators were modest, and it was not measured in some of the phase III trials. The position of sweat chloride as a biomarker of treatment response remains to be fully clarified, but the difficulty defining clinically meaningful threshold values, may limit its clinical utility as a biomarker.

As we assess the progress to date on CFTR modulation, we have witnessed success in establishing effective therapies for different subsets of patients with CF. It therefore

follows that there is a clear need for robust biomarkers which would enable clinicians to choose the appropriate modulators for each individual.

Conventional examination of genotype also has pitfalls, as studies to date have shown a heterogeneous response even amongst patients with identical genotypes. In addition mutations that have responded *in vitro* have not universally responded to modulation in trial settings as is evidenced by the lack of response to individuals carrying the G970R mutation (34).

Future identification of robust markers of CFTR function for each individual would enable clinicians to identify which therapy an individual may be most responsive to. This is a development which would enhance economical utility of these high cost medications (103, 104). This ultimately may enable the formation of specific therapy responsive classes of patients to supersede the conventional mutation classes. Recently published work has identified the measurement of forskolin induced swelling of intestinal organoids as a potential functional CFTR assay (105, 106). This measure appears to be responsive to CFTR modulator therapy and has shown a heterogeneous response amongst similarly genotyped individuals as is presently apparent clinically.

Conclusion

CFTR modulation, though in its infancy, is demonstrating multiple different effective approaches in CF. The realisation of the ability of restore CFTR function in the lung presents the question of the effect this will have on the traditional progression of CF lung disease namely decline in FEV_1, progressive bronchiectasis on chest CT, development of chronic *Pseudomonas aeruginosa* and whether CFTR modulation offers

the opportunity to reverse this process. Additionally, it highlights the question of which outcome measures one should use in evaluating treatment effectiveness and efficacy in a chronic illness and what constitutes benefit.

Given the multi-system nature of CF it also raises the possibility of whether CFTR modulation can improve or slow the development of CF associated comorbidities including endocrine and exocrine pancreatic disease, and gastrointestinal manifestations. The clear goal is to restore functional CFTR protein to all patients. Given that some rare mutations affect limited numbers of patients n-of-1 studies to consider the benefit to an individual patient are necessary. Equally the potential of using alternative methods of evaluating drug utility such an organoid technology other than expensive randomised clinical trials needs to be considered.

Aims and hypothesis

- **Hypothesis 1**: Clinical trials have strict inclusion and exclusion criteria; hence, we hypothesise that the real-world effectiveness of ivacaftor may be less than that observed in clinical trials. Larger numbers of patients with CF are reaching adulthood with relatively preserved lung function. As a result, we hypothesise that forced expiratory volume in one second (FEV_1) may not be the sole exclusive outcome measure dictating treatment success and there may be other important outcome measures.

- Aim 1 (Chapter 2): To evaluate the real-world effectiveness of restoration of CFTR function on standard clinical outcome measures including FEV_1, body mass index, sweat test, patient reported symptoms, pulmonary exacerbation frequency.

- **Hypothesis 2**: Ivacaftor enhances airway surface liquid and mucociliary function in vitro, thus we hypothesise that CFTR modulation may reverse the underlying lung pathology in CF including bronchiectasis, and lung microbiota disturbance.
  - Aim 2a (Chapter 3): To evaluate the effect of restoration of CFTR function on structural lung disease using ultra low dose chest computed tomography (CT).
  - Aim 2b (Chapter 3): To evaluate the effect of restoration of CFTR function lung microbiota, sputum *Pseudomonas aeruginosa* status and blood anti-*Pseudomonas aeruginosa* antibodies.
- **Hypothesis 3**: CFTR is expressed ubiquitously, consequently we hypothesise that ivacaftor may improve CF comorbidities including blood inflammatory markers, GI and pancreatic manifestations.
  - Aim 3a (Chapter 4): To evaluate the effect of restoration of CFTR function on circulating blood inflammatory markers and their correlation with clinical parameters.
  - Aim 3b (Chapter 4): To evaluate the effect of a restoration of CFTR function on exocrine and endocrine pancreatic comorbidity, namely pancreatic insufficiency as evaluated by faecal elastase, lipid profile and glucose tolerance.

- Aim 3c (Chapter 5): To evaluate the effect of a restoration of CFTR function on the CF gut comorbidities; including the gut microbiota, and markers of gut inflammation.

- **Hypothesis 4**: There are over 1800 CF associated mutations representing a spectrum of disease severity. Given that clinical trials have strict inclusion and exclusion criteria, the benefit derived by an individual patient may be different to the average response observed in clinical trials.
  - Aim 4a (Chapter 6): To evaluate the benefit of a restoration of CFTR function to the individual patient in a series of case studies.
  - Aim 4b (Chapter 6): To evaluate the relationship between forskolin induced intestinal organoid swelling and clinical response in patients with CF treated with CFTR modulatory therapy.

Chapter 2

The effect of CFTR modulation on standard clinical outcome measures

Introduction

The G551D mutation is the commonest class III mutation with a worldwide prevalence of 4-5% (8). However, there is significant regional variation (Figure 2.1) and prevalence of the G551D mutation in Cork Cystic Fibrosis Centre is 23%, making it uniquely placed to offer a single centre insight on treatment response (11).

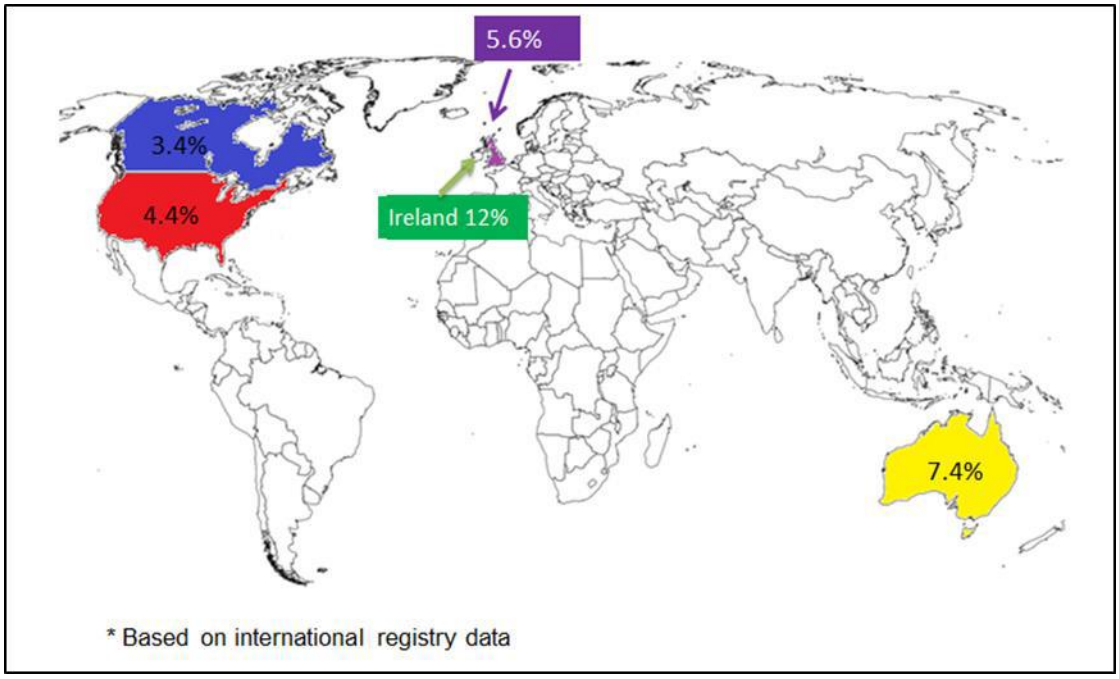


Figure 2.1 Worldwide prevalence of the G551D mutation

Clinical trials have demonstrated the clinical benefit of ivacaftor; however, real-world studies have demonstrated a smaller improvement in lung function than that observed in a clinical trial setting (23). Given the high prevalence of the G551D mutation in Cork the aim of this chapter is to evaluate the impact of ivacaftor on traditional clinical parameters – FEV_1, sweat test, body mass index, pulmonary exacerbation rate and patient reported outcomes - in a real-world setting and to assess tolerability and side effects.

Standard outcomes measures

Spirometry

FEV_1 is an important clinical parameter in patients with CF and the most frequently used primary endpoint in CF studies, as the first pulmonary defect in CF is obstructive in nature (107). The frequent use of FEV_1 as a primary outcome measures in CF studies is not surprising given that it reflects disease severity and progression. A lower FEV_1 is associated with an increased mortality and poorer quality of life (108, 109). Similarly, spirometry can be performed relatively easily and cheaply by most patients aged 6 and older and equipment is readily available.

Sweat testing

CFTR is expressed in the sweat glands. CFTR dysfunction typically results in abnormally high sweat chloride content. Sweat testing is performed to aid in the diagnosis of CF. An electrode is placed over pilocarpine disc on the persons skin. A second electrode is placed on the skin and the resultant electric current causes sweating. The site is cleaned and dried and the sweat is collected from the red electrode for 30 minutes. The chloride content of the sweat is then measured with diagnostic thresholds summarised in table 2.1. The clinical trials STRIVE and ENVISION demonstrated significant improvements in sweat chloride after ivacaftor (20, 21). Due to the expense associated with ivacaftor treatment the National Health Service (NHS) in England instigated a monitoring system and stopping criteria for ivacaftor treatment (99). The program requires that a sweat test be performed within 6 months of commencing

ivacaftor and after 8 weeks of treatment. Patients are regarded as having benefited from treatment if the sweat chloride post treatment falls to less than 60 mmol/l or by at least 30% (99). In patients with a pre-treatment sweat chloride less than 60 mmol/l, they are regarded as benefiting from treatment if the sweat chloride falls by at least 30% or if the patient has a sustained absolute increase in FEV_1 of 5% of more after one and three months of treatment (99).

Table 2.1 Sweat chloride diagnostic threshold Chloride diagnostic threshold	
< 40 mmol/L	Normal (CF very unlikely)
40 – 59 mmol/L	Intermediate (CF possible)
>60 mmol/L	Abnormal (CF likely)

Body mass index

In CF underlying lung disease gives rise to an increased resting energy usage (110). While impaired lung function and infection play a role in this, it has also been postulated that CFTR dysfunction itself also contributes to this increased resting energyusage (110). This in addition to pancreatic insufficiency and resultant fat malabsorption and recurrent pulmonary exacerbations may give rise to poor nutritional status. Low BMI is a poor prognostic indicator in CF with one study demonstrating that patients with CF with a BMI within the normal range had a higher FEV_1 % predicted than those with poor BMI; with the FEV_1 % predicted being on average 21.6% higher in the cohort with the normal BMI (111). Additionally, low BMI

has been demonstrated to be associated with increased risk of mortality in patients with CF referred for lung transplantation (112). Consequently, it is an important outcome measure in CF.

Pulmonary exacerbation

A high pulmonary exacerbation frequency is associated with an accelerated decline in lung function and increased morbidity in CF (113, 114). In approximately 25% of patients' lung function does not recover to baseline after a pulmonary exacerbation (113). Pulmonary exacerbations are also associated with reduced quality of life and accelerated decline in lung function (115, 116). Minimising the number of pulmonary exacerbations in patients with CF is an important aspiration in preserving lung function and enhancing quality of life and represents an important endpoint in the treatment of CF. There is no consensus on what constitutes a pulmonary exacerbation in CF and a wide variety of definition of pulmonary exacerbation criteria have been utilised in various studies including EPIC, Fuchs, Azithromycin trial and ISIS trial criteria (117-120). Table 2.2 summarises some the definitions utilised in various criteria. Given the variability in diagnostic criteria for a pulmonary exacerbation we evaluated the impact of ivacaftor on patient's requirement for intravenous antibiotics as this requires physician review and prescription.

Table 2.2 Pulmonary exacerbation criteria
EPIC Criteria – presence of one major or two minor **Major criteria:** 1. Decrease in FEV_1 ≥10% from best baseline in past 6 months, unresponsive to albuterol 2. SaO2 < 90% on room air or a decline of ≥5% from previous baseline 3. New lobar infiltrate/atelectasis on chest x-ray 4. Haemoptysis (more than streaks on more than one occasion in the past week) **Minor criteria:** 1. ↑work of breathing or respiratory rate 2. New or increased adventitial sounds on lung exam 3. Weight loss ≥5% of body weight or decrease across 1 major percentile in weight percentile for age in past 6 months 4. Increased cough 5. Decreased exercise tolerance or level of activity 6. Increased chest congestion or change in sputum Duration of signs/symptoms ≥5 days or significant symptom severity
Fuchs Criteria – Presence of 4, resulting in IV antibiotic prescription 1. Temp >38°C 2. Anorexia/weight loss 3. Sinus pain/tenderness 4. Change in sinus discharge 5. Change in chest exam 6. Decrease in FEV_1 ≥10% **7.** Radiographic changes

Patient reported outcomes

Patient reported outcomes (PRO's) are self-reported feedback recorded from a patient on how they feel or function; recorded by means of a validated questionnaire or diary. They give an insight into the patient's perception of treatment and measure the treatment benefit to the patient beyond survival, physiological or biochemical markers (121). There has been an increasing trend towards the use of PRO's as an outcome measure in clinical trials. An EMA workshop on endpoints in clinical trials in CF agreed

that the best validated tool to assess PRO's in CF is the cystic fibrosis questionnaire revised (CFQ-R) (107, 122). The minimally clinically important difference (MCID) for the CFQ-R respiratory domain has been established as 4.0 in clinically stable patients and 8.5 in those with a pulmonary exacerbation. It has previously been used in clinical trials of inhaled antibiotics, dry powder antibiotics, and ivacaftor with a focus on the respiratory domain (107). However, there are some limitations. The CFQ-R was originally developed to measure the impact of CF on quality of life as opposed to foruse as a clinical trial PRO measure. CFQ-R has additional physical, vitality, emotion, eating, treatment burden, health perception, social, body image, role, weight anddigestion domain, allowing multi-system assessment of symptoms (122).

Methods

Patients with CF aged six and older with at least one copy of the G551D mutation attending Cork Cystic Fibrosis centre attended from March 2013 and followed prospectively, for a mean period of follow up of 12 months. The prevalence of the G551D mutation at Cork CF Centre is 23%, with 51 of 220 patients carrying at least one copy of the G551D mutation. Thirty-three patients with the G551D mutation consented to participate. Eight children were too young to receive ivacaftor, six patients had already commenced ivacaftor either as part of a clinical trial or on a named patient (compassionate use) basis, two patients were post-lung transplantation,and three were undecided as to whether they wished to start treatment. According to the UK CF Trust annual report of 2015, there were 32 patients with the G551D

mutation in Northern Ireland, representing 8% of the CF population. According to the CF Registry of Ireland annual report, there are 93 patients with the G551D mutation in the Republic of Ireland, representing 11.3% of the CF population. Thus, this patient group represents approximately one-quarter of patients with the G551D mutation on the island of Ireland. Ethical approval was obtained from the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Ivacaftor-naive patients with CF attended for assessment when clinically stable and free from pulmonary exacerbation, at which point written informed consent to participate was obtained. Spirometry was performed according to ERS/ATS guidelines using CareFusion MicroLab™ spirometer which is calibrated on a regular basis (123). Sweat testing was performed using a Macroduct™ system in accordance with manufacturer's guidelines. Patients aged 14 and older completed Cystic Fibrosis Questionnaire Revised (CFQ-R) and for patients aged 6 -14 parents completed Care- giver CFQ-R (122). Participants were assessed on a three-monthly basis after commencement of treatment. The numbers of courses of intravenous antibiotic (IV) forpulmonary exacerbations were recorded prospectively for 12 months after ivacaftor and compared with the number of courses of IV antibiotics 12 months before commencing ivacaftor.

Statistical Analysis

Data was analysed using SPSS version 22·0 and STATA. A mean post follow-up value was calculated for each parameter for each patient (mean of available 3, 6, 9- and 12-month values) and this post value was compared to the value before commencing

ivacaftor. Paired sample t test was used to evaluate mean change from baseline for normally distributed variables. Paired Wilcoxon signed rank test was used to evaluate median change in non-normally distributed data. Pearson correlation was performed to evaluate the relationship between FEV_1 and sweat chloride and CFQ-R respiratory domain.

Results

Clinical

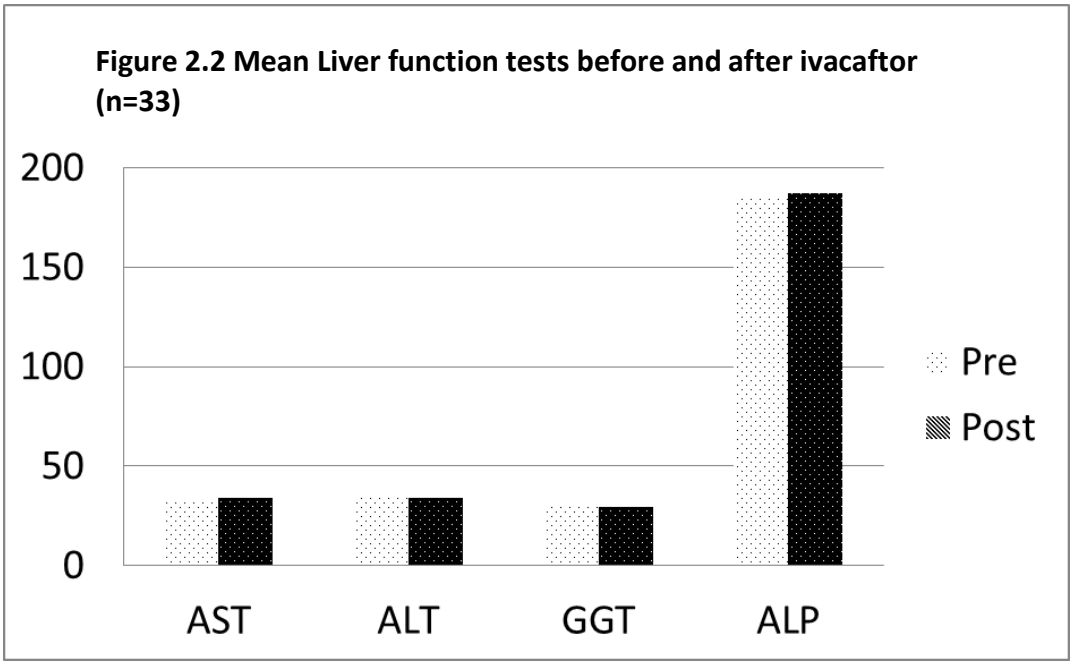
Twenty adult (age ≥16) and 13 paediatric (age 6 – 16) patients participated. Table 2.3 summarises baseline characteristics.

Table 2.3 Baseline Characteristics	Mean (SD) N = 33
Gender (% male)	70
Age (years)	21.6 (5.4)
FEV_1 (% predicted)	75.21 (20.7)
Weight (Kg)	51.26 (19.7)
BMI (Kg/m^2)	19.46 (3.5)
Sweat test (mmol/l)	101 (14.7)
Mutation (%)	
G551D/F508del	85%
G551D/G551D	3%
G551D/3028delA	3%
G551D/1717-1G→A	3%
G551D/Glu56Lys	3%
G551D/R553X	3%

The mean age of the cohort was 21.6 years and 70% of participants were male. The mean baseline FEV_1 % predicted was 75.21% (*SD 20·7*). Mean baseline sweat chloride

was 101 mmol/l (*SD 14·7*). The majority (85%) of patients had the F508del mutation as their second mutation. One patient was homozygous for the G551D mutation. All other patients had a class I or II mutation as their second mutation. No significant derangement of liver function tests (LFT's) (including bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyltransferase (GGT)) were observed in the cohort (Figure 2.2).

Figure 2.2 Mean Liver function tests before and after ivacaftor (n=33)



After commencement of therapy a 10·3% mean increase in FEV_1 % predicted was observed (*p<0·001*) (Figure 2.4). A 58 mmol/l mean reduction in sweat chloride was observed after treatment (*p<0·001*) (Figure 2.3).

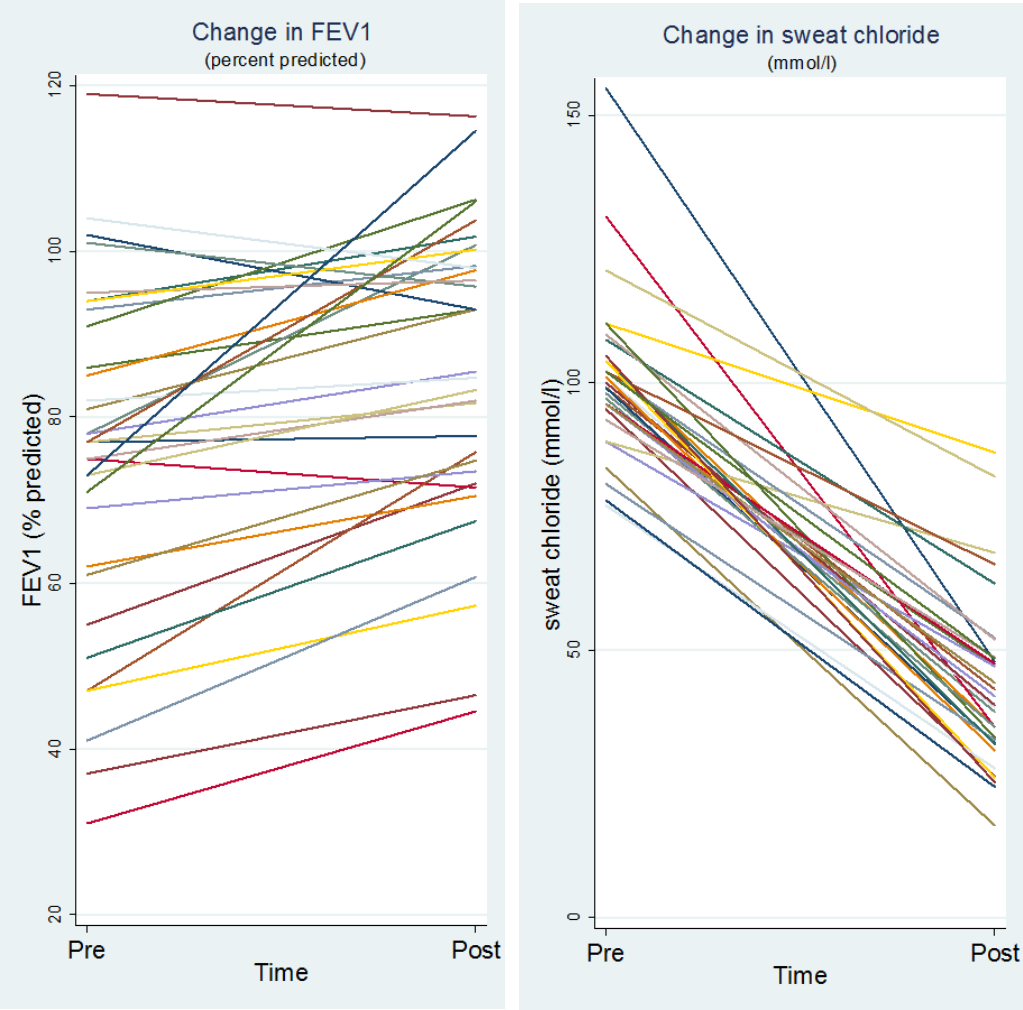


Figure 2.3 FEV_1 % predicted and sweat chloride before after ivacaftor (n=33)

A 1.2 kg/m^2 significant mean increase in BMI (*p<0·001*) and a 4 kg significant mean increase in weight (*p<0·001*) were observed after commencement of ivacaftor (Figure 2.4).

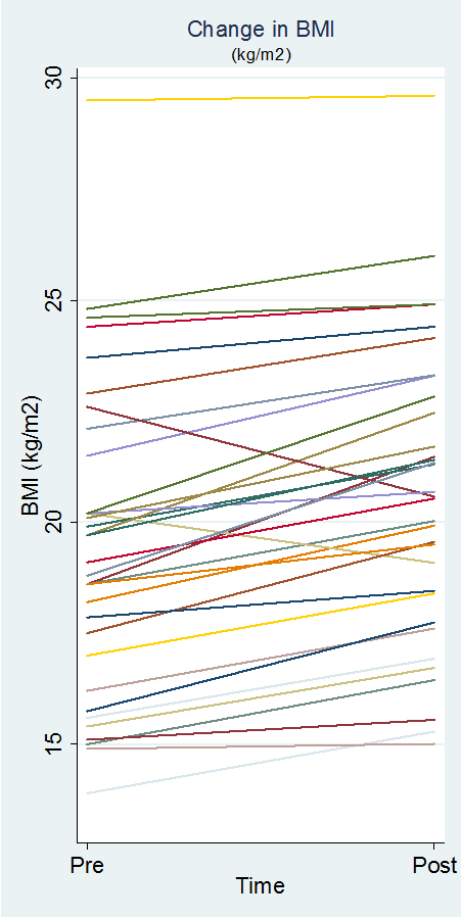
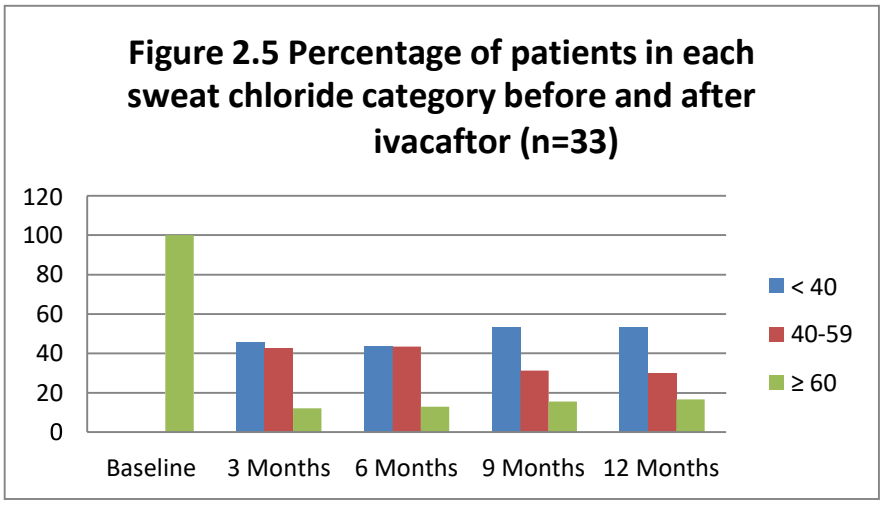


Figure 2.4 BMI before and after ivacaftor (n=33)

A 76 % reduction in pulmonary exacerbations requiring intravenous antibiotics was observed in the first year of therapy compared to the year before treatment (*p=0·006*). After 3 months 88% of patients had a sweat chloride below the level considered diagnostic for CF (60 mmol/l) (Figure 2.5). Of the 4 patients who had a sweat chloride

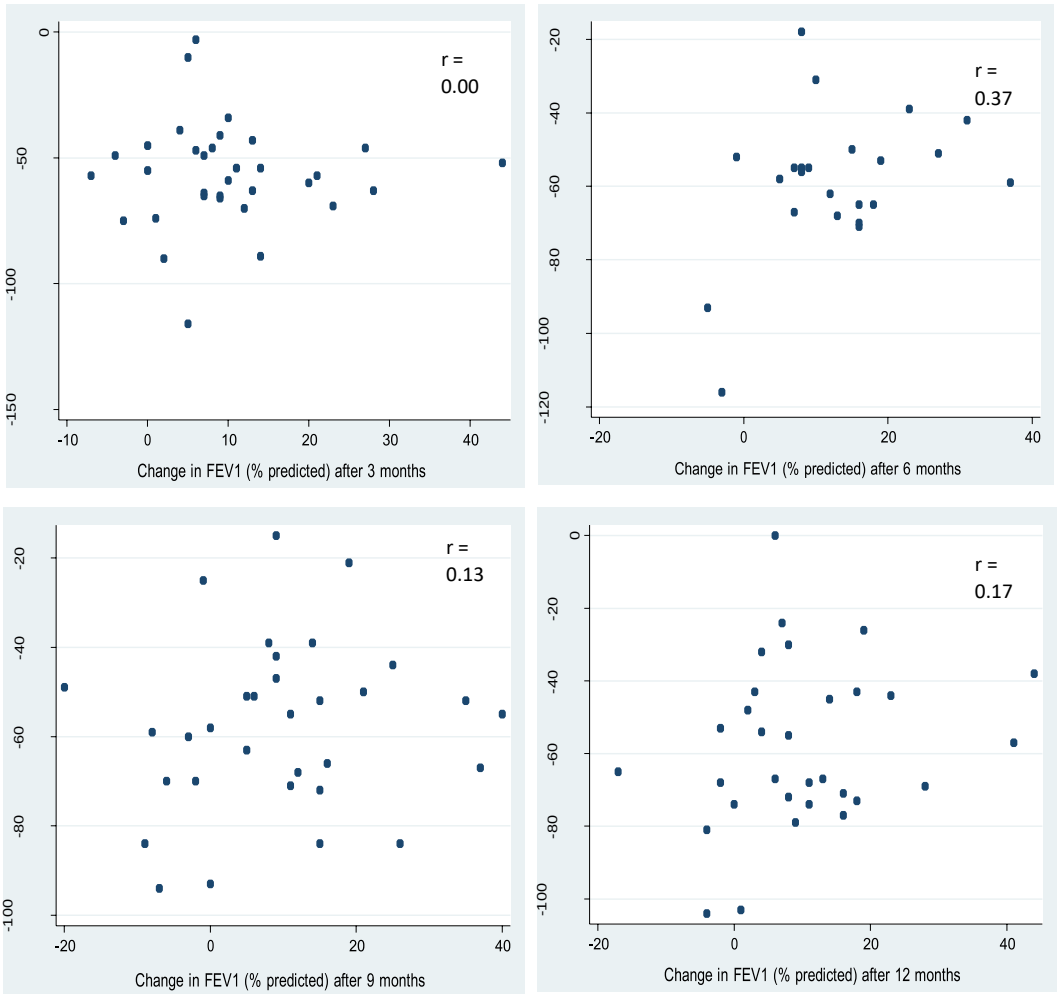
greater than 60 mmol/l after three months of treatment two patients did not have a fall in sweat chloride of at least 30%.

Patient 1 had a sweat chloride of 89 mmol/l at baseline and 86 mmol/l after three months. Patient 2 had a sweat chloride of 111 mmol/l at baseline and 101 mmol/l after three months. Despite this lack of sweat chloride response, the patients experienced a 6% and 5% absolute increase in FEV_1 after three months of treatment respectively.



No significant relationship was observed between the magnitudes of change in FEV_1 and the magnitude of change in sweat chloride after 3, 6, 9 or 12 months of treatment.

Figure 2.6 Relationship between change in FEV_1 and change in sweat (n=33)



A 17·5-point mean increase in Adult CFQ-R Respiratory Domain (*p<0·001*) and an 8.8-point mean increase in caregiver completed CFQ-R Respiratory Domain (*p=0·08*) were observed (Figure 2.7).

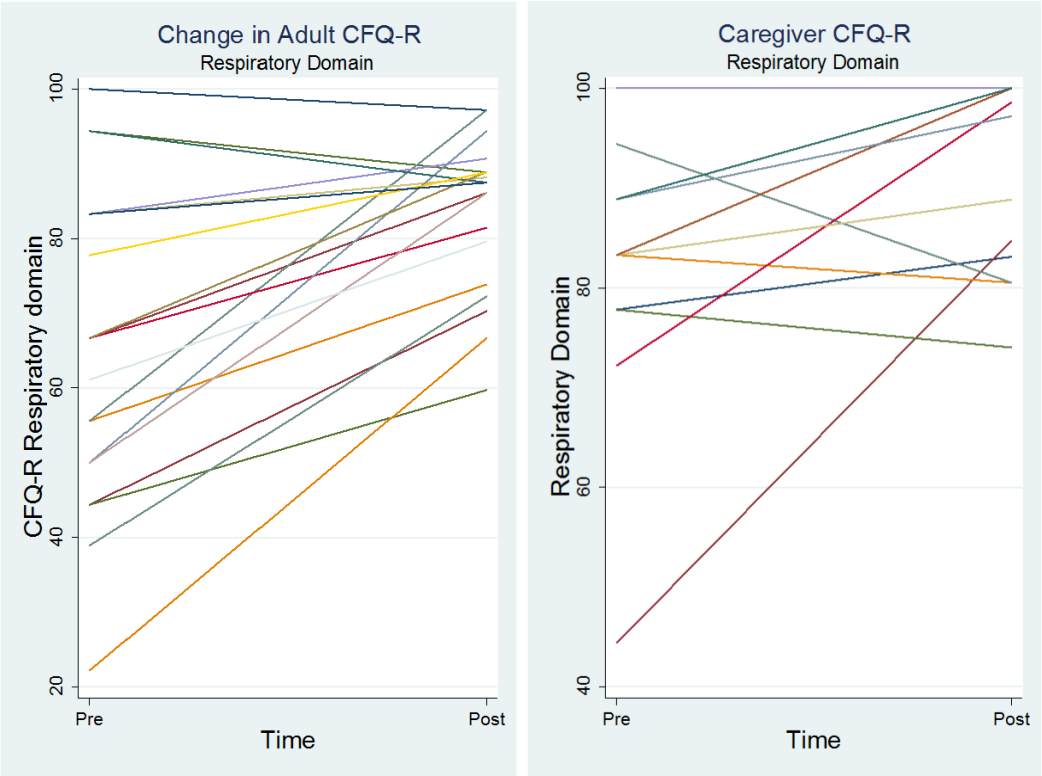


Figure 2.7 Adult and caregiver CFQ-R Respiratory Domain before and after ivacaftor

Changes in other domains of the adult CFQ-R are summarised in Table 2.4. A statistically significant improvement in the weight domain was observed after treatment.

Table 2.4 Mean Adult CFQ-R Before and After Ivacaftor (N = 20)

CFQ-R Domain	Mean baseline score (SD)	Mean change (SD)	*p value*
Eating	92.78 (15.8)	2.036 (8.7)	*0.312*
Physical	86 (17.6)	4.35 (12.6)	*0.139*
Vitality	66.25 (15.87)	5.5 (13.5)	*0.082*
Emotion	79.76 (15.6)	2.4 (7.88)	*0.188*
Treatment burden	67.24 (18.56)	5.6 (14.9)	*0.109*
Health perception	74.46 (19.78)	6.1 (18.7)	*0.161*
Social	78.33 (14.95)	0.88 (12.78)	*0.761*
Body Image	69.46 (25.21)	8.8 (22.17)	*0.092*
Role	86.26 (18.2)	1.66 (8.66)	*0.402*
Weight	58.34 (37.27)	22.63 (31.22)	*0.004*
Digestive	86.12 (16.69)	3.06 (10.78)	*0.22*

Changes in other domains of the parent/ caregiver CFQ-R are summarised in table 2.5. Significant improvements in the body image and weight domain were observed.

Table 2.5 Mean Parent/caregiver CFQ-R Before and After Ivacaftor (N = 11)

CFQ-R Domain	Mean baseline score (SD)	Mean change (SD)	*p value*
Physical	94.96 (5.6)	1.74 (8.42)	*0.489*
Emotion	85.5 (12.3)	1.26 (12.79)	*0.739*
Vitality	73.3 (17.75)	1.11 (10.67)	*0.725*
School	78.73 (17.36)	2.38 (13.3)	*0.548*
Eating	86.11 (18.57)	-0.236 (19.26)	*0.967*
Body Image	75.9 (28.76)	13.73 (18.65)	*0.027*
Treatment Burden	62.97 (21.38)	-2.0 (17.5)	*0.7*
Health perception	84.28 (12.9)	-0.7 (18.69)	*0.899*
Digestive	80.58 (16.48)	7.7 (15.5)	*0.113*
Weight	63.89 (33.22)	21.76 (33)	*0.043*

Discussion

We observed significant improvements in lung function, weight, and CFQ-R after commencement of ivacaftor treatment. The improvement in lung function was in keeping with that observed in the clinical trials and larger than that observed in other observational studies in people receiving treatment outside of clinical trials (23). This may be due to the fact that our cohort had a lower baseline FEV_1 than those in other observational studies (23). However, despite its widespread use there is an awareness that FEV_1 is not without its limitations. Depending on the equation used there may be variability in a person's FEV_1, in particular during a period of growth (124, 125). Additionally, with incremental improvements in CF care larger numbers of patients are

reaching adulthood with relatively preserved lung function (126). This may pose problems in using FEV_1 as a primary outcome in clinical trials in the future given that larger numbers of patients will be required to demonstrate a clinical benefit. Additionally in patients with relatively preserved lung function it may be more difficult to demonstrate an FEV_1 improvement in a therapy that has long-term benefit (107). Lastly FEV_1 may be insensitive to subtle clinical deterioration. Disease progression has been on chest CT and using lung clearance index in patients who's FEV_1 has remained stable over time (127). Thus, it may be a sub-optimal outcome measure for a treatment which addresses the underlying CFTR defect.

The improvement in the respiratory domain of the CFQ-R was larger than that observed in the clinical trials which may also be reflectively of a greater perceived symptom improvement in patients with lower lung function (20, 21). There was no significant change in other domains of the CFQ-R. It is notable that there is a ceiling effect in patients with milder disease who may achieve the maximum score in an individual domain and this represents one of the limitations of CFQ-R (107). Notwithstanding that, patient perceived benefit of a medication may be an important consideration in the development of new treatments due to the potential for better adherence to a medication from which a patient perceives a health and symptomatic benefit. The sustained improvement in BMI is important given the association of increased mortality in patients with CF with low BMI but raises the query of a need for long-term monitoring and the potential for development of obesity if a high fat diet is continued unchecked.

In keeping with a previous study no correlation was observed between the change in FEV_1 % predicted and change in sweat chloride levels at any of the time points, suggesting that it is a poor marker of treatment response and that the relationship between improvements in FEV_1 and sweat chloride are not linear (100). Similarly, in our cohort two patients failed to demonstrate a sweat chloride response, however, they both experienced an improvement in FEV_1 of 5% or more. This suggests that sweat chloride measurement is not a robust biomarker of pulmonary response to CFTR modulatory therapy in isolation.

The improvements in FEV_1 were paralleled by a significant 76% reduction in IV antibiotic requirements in the year after ivacaftor treatment. Given that a high pulmonary exacerbation rate has been shown to accelerate the decline in FEV_1, this real-world reduction in exacerbation rate as it may contribute to reducing annual rate of decline in FEV_1 in patients treated with CFTR modulators. This would be an important long-term benefit of CFTR modulatory therapy and must factor into an analysis of treatment benefit. However, importantly it must be born in mind that there is variability and a lack of consensus on the definition of a pulmonary exacerbation (118, 128). Frequently the definition of a pulmonary exacerbation is dependent on patient reported symptoms and a physician's decision to treat with intravenous antibiotics therefore, raising the potential for introduction of bias which may be a potential limitation in the utility of pulmonary exacerbation frequency as an outcome measure in CF. A further important consideration in CF studies is pulmonary exacerbation frequency may be low in people with "milder" disease and in young

children necessitating larger numbers of participants in a study to demonstrate a significant difference (129, 130).

Notwithstanding the real-world effectiveness of ivacaftor in this real-world cohort, among the limitations of these classically utilised outcome measures in CF are the fact that they are most useful in patients who have impaired pulmonary function. They may not be the best outcome measures in people who have relatively preserved pulmonary function. Given that CFTR modulators treat the underlying cause of CF and potentially are a disease modifying therapy which may slow or prevent the development of classic CF associated morbidities additional outcome measures in addition to those listed above may be needed to assess its true utility.

Limitations

A limitation of this study is that it is observational in nature without a control group who did not receive ivacaftor treatment. However, the benefits of ivacaftor relative to placebo have been well demonstrated in existing trials; the observed improvements are similar to those reported in the trials and would be disproportionate to changes expected in an untreated cohort. We avoided recruitment sample-related variability given that we included the entire cohort of patients attending our designated referral centre for the region. All eligible patients received the drug, as it was funded by the state and not dependant on health insurance status.

Conclusion

This study demonstrates sustained real-world effectiveness of ivacaftor treatment across standard assessment parameters. In keeping with previous studies, it suggests that sweat chloride in isolation is not a useful marker of treatment response.

Chapter 3

The effect of CFTR modulation on novel clinical outcome measures

Introduction

As the treatment of CF moves from an era of treating the consequences of CFTR dysfunction to treating the underlying cause the need for additional outcome measures in patients with CF becomes apparent. While historically the expectation was that patients with CF would reach adulthood with significant pulmonary impairment, the improved care, recognition of milder phenotypes and CFTR modulators has challenged this. Given the difficulties in detecting a significant change in FEV_1 in patients with relatively preserved lung function and the additional limitations discussedin relation to standard outcome measures in chapter 2, this chapter aims to evaluate the utility of novel and emerging outcome measures.

Exercise Field Testing

Formal exercise testing can be performed in a laboratory setting. Informal field testing including modified shuttle walk test and six-minute walk test are inexpensive and efficient methods for quantifying exercise capacity (131, 132). The use of formal exercise field testing as an outcome measure in clinical trials in CF is somewhat limited.However, there are numerous previous publications demonstrating an association between exercise capacity and survival in patient with cystic fibrosis (133-135). Hence, there is reason to postulate that it would be a useful outcome measure in CF studies. Equally field testing in the form of six-minute walk test has been used in patients with other progressive chronic lung conditions including idiopathic pulmonary fibrosis (IPF) and pulmonary hypertension clinical trials and been demonstrated to be a reliable,

valid and responsive marker to treatment status (136, 137). This raises the question of the utility of field exercise testing as an outcome measure for new treatments for people with CF.

Chest Computed Tomography (CT)

There is evidence that airway infection, inflammation and structural lung disease may be present in patients with CF even in the absence of overt respiratory symptoms (138, 139). In CF lungs, recurrent bacterial infection and excessive inflammation give rise to structural lung damage, including bronchiectasis. Consistent with this excessive inflammatory response, airway wall thickening is observed in approximately 85% of patients with CF and is more severe in the peripheral airways as opposed to central airways (140, 141). Over time as a result of chronic inflammation airways become abnormally dilated or bronchiectatic. CF is typified by extensive bilateral bronchiectasis with upper lobe predominance. Bronchiectasis is considered to be progressive and irreversible. The AREST CF study demonstrated that bronchiectasis develops in very early life in CF with an incidence of 8.5% in the first year of life and can be observed on CT chest as early as 3 months of age (142). In a study of 96 young children with CF who did not have symptoms up to 22% had evidence of bronchiectasis and 20% had evidence of bronchial wall thickening (142). Up to 80% of patients with CF may have evidence of mucus plugging on chest CT (143). This develops as a result of a variety of processes including reduced airway surface liquid and impaired mucociliary clearance.

Spirometry is less useful for detecting peripheral airway disease, therefore, normal spirometry may not reflect normal lung structure (144). Chest CT changes including mild bronchiectasis can be demonstrated in people with CF who have a normal FEV_1. Inone study of children aged 6 to 10 years old 30% of children with normal PFT's (FEV_1 >85%) had evidence of bronchiectasis on chest CT (145). In work involving patients with CF aged 12 years and older who had an FEV_1 > 90% predicted, 73% had evidence of bronchiectasis. Equally disease progression has been observed on CT scanning in individuals where FEV_1 has remained stable over time (143, 146). In a study involving 48 people with CF over a two year period, while HRCT scores disimproved significantly over time (irrespective of the CT scoring system used, Castile, Brody, Helbrich, Santamaria and Bhalla) spirometry did not change significantly (143). There was a significant correlation between the HRCT score and FEV_1 at baseline and after two years but a weak correlation between the magnitude of change in HRCT score and the magnitude of change in the FEV_1 over time. It was mainly mucus plugging, and the severity, extent and peripheral extension of bronchiectasis which contributed to the CTprogression over time (143). As a result, there is awareness that FEV_1 may not be the optimal marker for monitoring disease progression over time particularly in people with "mild" lung disease and that chest CT may be a more sensitive marker. Chest radiographs also appear to be insensitive to acute changes in people with CF (147).

CT has previously been used as an outcome measures in a double-blind randomised placebo-controlled trial of dornase alpha in children with mild CF lung disease, with chest CT and spirometry being used to assess treatment response. In the children

treated with dornase alpha a composite CT/PFT score had a better ability to detect change from treatment than did either alone (148). More recent work has demonstrated PRAGMA-CF CT score to be reproducible and to correlate with neutrophil elastase in children younger than 6 with cystic fibrosis (149).

A single study has demonstrated improvement in chest CT and MRI post ivacaftor, mostly due to changes in mucus plugging (77, 82). However, in the small cohort evaluated with chest CT, no significant improvement in FEV_1 was observed during treatment with ivacaftor and the study used standard dose Chest CT (mean effective radiation dose of 3.6mSv per scan) (77). There is a growing awareness that patients with CF are at increased risk of certain neoplasms including those involving the gastrointestinal tract (150). With this in mind this chapter aims to use a previously validated low dose Chest CT protocol (mean effective radiation dose of 0.08mSv per scan) to evaluate response to ivacaftor and to correlate this will clinical parameters. This may be an acceptable method of monitoring treatment response.

Lung microbiota

CF is typified by recurrent pulmonary infections and an exaggerated inflammatory response with a consequent decline in pulmonary function. The role of pulmonary infection in contributing to morbidity and mortality in CF has been recognised as far back as 1938 when Dorothy H Andersen reported on bronchopulmonary infection with *Staphylococcus aureus* in autopsy samples from patients with CF (151). Regular bacterial culture of sputum samples from patients with CF at clinic visits has been part

of routine management for many years. Bacterial colonisation of the respiratory tract develops early in patients with CF (152-154). Typical CF associated pathogens include *Staphylococcus aureus*, *Haemophilus influenza, Pseudomonas aeruginosa, Methicillin Resistant Staphylococcus aureus (MRSA), Achromobacter xylosoxidans, Stenotrophomonas maltophilia* and *Burkholderia cepacia complex* (Figure 3.1).

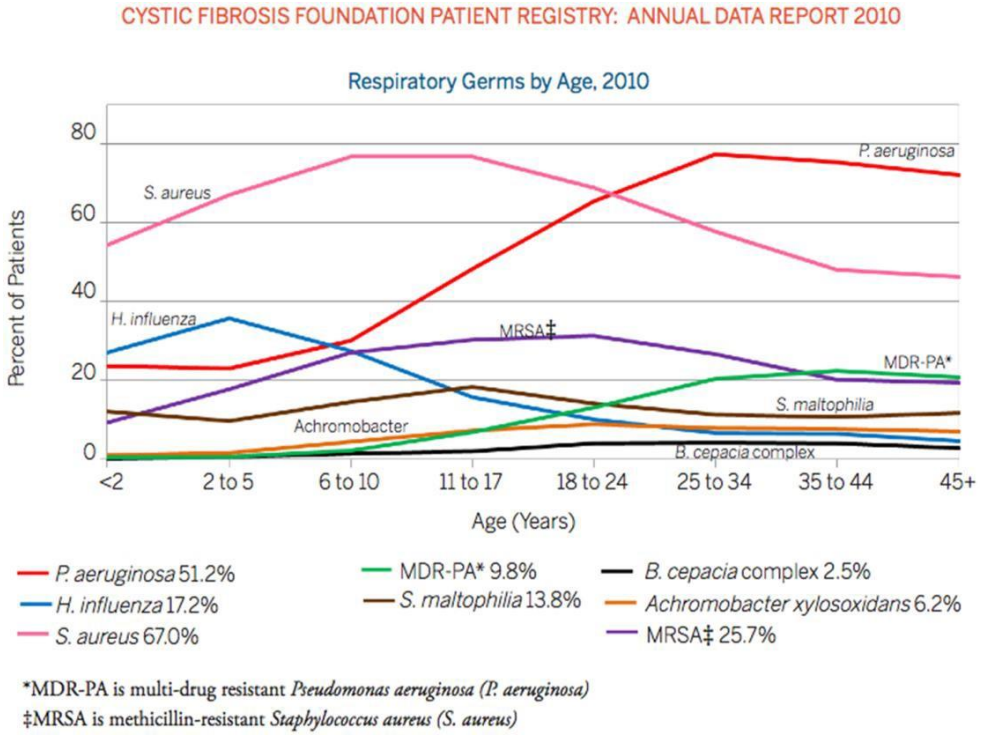


Figure 3.1. CF Lung microbiome evolution with age (Cystic Fibrosis Foundation Patient Registry: Annual Data Report 2010)

Pseudomonas aeruginosa colonization increases with time in patients with CF, with some studies estimating that up to 80% of adults with CF are chronically colonized withthis organism (155). *Pseudomonas aeruginosa* was the most common respiratory pathogen in Irish patients with CF aged 20 and older in 2014 with prevalence ranging

from 50 – 71.1% depending on age category (156). It is well established that chronic pulmonary colonisation with *Pseudomonas aeruginosa* is associated with increased pulmonary exacerbation frequency and accelerated decline in FEV_1 (111) . CF guidelines recommend attempted eradication of this organism after initial colonisation and chronic suppressive inhaled or nebulised antibiotics in those who are chronically colonised with *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* may be successfully eradicated in the early stages, however, with time *Pseudomonas aeruginosa* changes from a non-mucoid to mucoid phenotype which gives rise to biofilm formation (157, 158). Biofilm formation may then result in reduced susceptibility to antibiotic killing (158). The increasing availability of anti-Staphylococcal and anti-Pseudomonal antibiotics has contributed to increased longevity in patients with CF, highlighting the important role of the lung microbiota in CF.

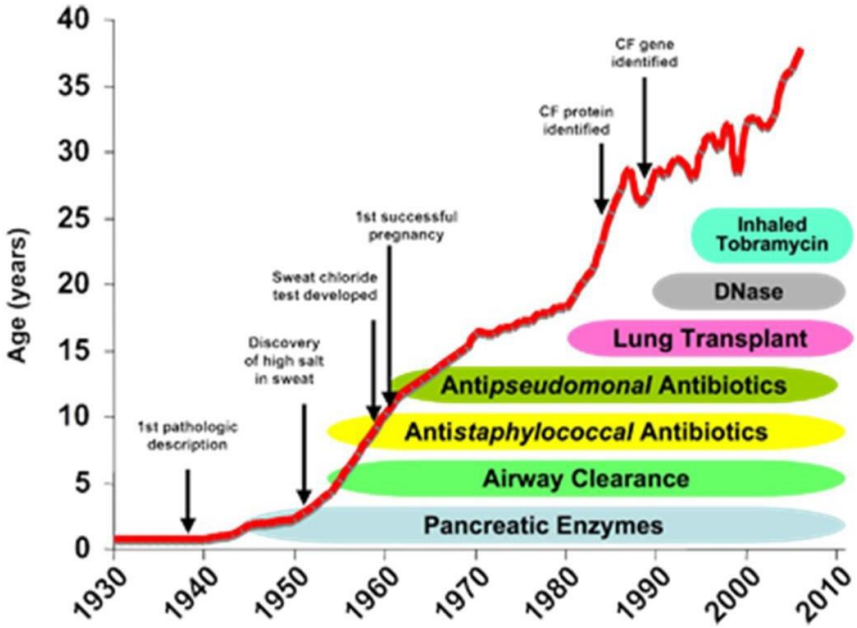


Figure 3.2 Evolution of CF therapies

The European Medicines Agency guideline on the clinical development of medicinal products for the treatment of cystic fibrosis suggest that a microbiological primary endpoint at 28 days is an acceptable primary endpoint for confirmatory trials in the treatment of early lung colonisation or of chronic infection exacerbations (107). Given that ivacaftor enhances CFTR function we aim to assess its impact on the CF lung microbiota.

Culture independent assessment of lung microbiota

The emergence of culture-independent methods for identifying organisms present in the CF lung has given a greater insight into the complexity and variety of bacteria present in the CF lung. The increasing availability of culture independent methods for detecting bacteria in sputum samples has demonstrated that the lung microbiota is more complex than initially recognised (159). While sputum culture work in CF suggest an average of 13 bacterial species in each sputum sample, next generation sequencing using 16S rRNA have demonstrated the presence of far larger numbers of organisms in the lung (160). An additional advantage of using 16S rRNA sequencing is that it allows for a quantification of bacterial diversity and relative abundances. Lung bacterial diversity seems to increase during the first 10 years of life and then decreases during adulthood as lung function begins to decline (161, 162). Lower lung bacterial diversity has been shown to correlate with lower lung function (161). Lastly, identification of anaerobic bacteria using standard culture is often challenging, requiring techniques and conditions which are not standard practice in many laboratories (163). By contrast

culture independent analysis of CF samples have highlighted the presence of anaerobic bacteria in the CF lung and is some instances at an abundance level equal to or exceeding that of *Pseudomonas aeruginosa* (160, 164, 165).

Diversity index is a numerical reflection of the number of different species present in a sputum sample. It also takes into consideration how evenly (evenness) the different species are distributed in the sample and the number of different species (richness) present. Two small studies have suggested changes in the lung microbiota after commencement of ivacaftor in keeping with a healthier profile (23, 166).

Anti-pseudomonas aeruginosa antibodies

Anti-Pseudomonas aeruginosa antibodies in blood have previously been demonstrated to be low at the time of initial colonization and to rise as a patient becomes chronically colonized with mucoid *Pseudomonas aeruginosa (167)*. They have been shown to fall with successful eradication of *Pseudomonas aeruginosa* with anti-Pseudomonaltherapy. It has been suggested that they may be more sensitive and specific for than respiratory sputum culture for evaluating the presence of *Pseudomonas aeruginosa (167-169)*. Given that some work has suggested a reduction in *Pseudomonas aeruginosa* after ivacaftor we evaluated the impact of ivacaftor on *anti-Pseudomonas aeruginosa* antibodies.

Methods

As previously discussed in chapter 2 patients were assessment at baseline and on a three-monthly basis after commencing ivacaftor. In addition to the standard clinical parameters a modified shuttle walk test was performed at each visit. In the shuttle walk test the patients were asked to walk between two markers which were spaced 10 metres apart. The pace was set by a beep and initially a very slow pace but subsequently became faster. The aim is to turn around a marker at the first beep and around the second marker at the next beep. As the test progressed the beeps come closer together and the pace becomes faster until the patient completed the test or could not continue due to dyspnoea. The test has a maximum of 15 levels, with each level having a duration of 1 minute (170).

Radiology

Adapted low dose chest CT were performed (mean effective radiation dose 0·08 mSv), using a previously validated protocol (171), at baseline and after 3, 6 and 12 months of treatment on consenting participants aged 16 and older. All studies were acquired using a 64-slice multidetector CT scanner (General Electric Discovery CT 750 HD; GE Healthcare, GE Medical Systems, Milwaukee, WI, USA) without intravenous contrast material. A modified 7-section, low-dose axial CT protocol previously validated at our institution was used for the pre-treatment and first 12-month quarterly studies. Single anteroposterior and mediolateral localizer radiographs were used to identify 5 levels, evenly spaced, at which images were acquired. Images were obtained with the patient

at end-inspiration through the lung apices, aorto-pulmonary window, carina, and at the widest cardiac and thoracic diameters. Two further images were obtained with the patient in full expiration at the aorto-pulmonary window and at the widest cardiac diameter. The following parameters were used: tube voltage of 120 kV; gantry rotation time of 0.4 seconds; field of view (FOV) of 32cm; and z-axis automatic tube current modulation with minimum and maximum tube current thresholds set at 10 and 100 mA with a tolerated noise index of 29HU. Images were acquired at each of the 7 levels at a slice thickness of 0.625mm and reconstructed to a slice thickness of 1.25 mm with the standard departmental protocol employing hybrid IR: 70% filtered back projection and 30% adaptive statistical iterative reconstruction (ASIR) (GE Healthcare, GE Medical Systems, Milwaukee, USA).

Quantification of lung disease

Disease severity was scored independently by two experienced consultant radiologists using a validated scoring system - Bhalla score (172). The presence and severity of 9 morphological changes were evaluated including:

1. Severity of bronchiectasis
2. Peribronchial thickening
3. Extent of bronchiectasis (number of bronchopulmonary segments)
4. Extent of mucus plugging (number of lung segments)
5. Abscesses or sacculation (number of lung segments)
6. Generations of the bronchial divisions involved
7. Number of bullae

8. Air trapping (number of lung segments) and
9. Collapse/consolidation.

A score of 0 to 3 (0: absent; 1: mild; 2: moderate; 3: severe) was assigned to each category to give a total score ranging from 0 to 25. A score of 0 indicated that no abnormality was detected.

Sputum

Expectorated sputum samples were collected during the baseline pre-treatment visit for the initiation of ivacaftor treatment and at the last POST-treatment visit (i.e. at 9 or 12 months). Fourteen patients provided sufficient sputum to allow culture- independent molecular analysis with a further 10 patients providing sufficient sputum to also allow processing by extended-quantitative bacterial culture. Sputum samples were stored at -80°C and shipped on dry ice to Queen's University Belfast where they were stored at -80°C until processing. 16S rRNA marker gene sequencing was performed on the Illumina MiSeq platform (Illumina, CA, USA) targeting the V4 the hyper-variable region of the 16S rRNA gene as previously described (173). qPCR quantification of total bacterial and *P. aeruginosa (PA)* density was determined by qPCR using the LightCycler®480 instrument (Roche, CH) using the Probes Master kit. Total bacterial load was determined using a primer/probe targeting the bacterial 16S rRNA marker-gene (174) , with *P. aeruginosa* load determined using a primer/probe set targeting the opr L gene (175).

Sputum processing for microbiological analysis

Frozen sputum samples were thawed, and any sputum plugs were separated from any saliva like material for analysis. For pre-lysis of sputum plugs transfer ~100mcg of

sputum to a sterile Eppendorf tube and mixed with an equal volume of 10% dithiothreitol (DTT, Sputolysin®, Calbiochem, CA, USA). Each sample was mixed by vigorous vortexing and incubated at room temperature (RT) for 30 minutes on a thermo-shaker at 2000 RPM. Next add 200 µl lysis buffer (5 mg/mL lysozyme in BLB Roche Bacteria Lysis Buffer]), vortex thoroughly and incubate for 30 minutes at 37°C inan orbital thermo-shaker at 2000 RPM. Transfer the total volume to a glass bead tubes (matrix A) and homogenize on the FastPrep®-24 instrument (MP Biomedical, CA, USA) at speed setting 6.0 for 40 seconds. Remove the sample tubes from the FastPrep®-24 instrument and centrifuge at 13,000 x g for 1 minute. To the homogenized sample add 32 µl of proteinase K (20 mg/mL, Qiagen, Hilden, Germany) and mix thoroughly by vortexing. Incubate at 65°C for 10 minutes on a heated thermo-shaker at 1500 RPM. Next add 150µl of nuclease free water, place the tube in the FastPrep®-24 instrument (MP Biomedical, CA, USA) and homogenize at speed setting 6.0 for 40 seconds. Incubate the homogenized sample at 95°C for 10 minutes on a heated thermo-shaker at 1000 RPM. Finally, centrifuge at 10,000 x g for 10 minutes at 4°C and transfer 200 µl to a sterile Eppendorf tube for storing at -80°C until further use.

Extraction of gDNA from sputum samples

Pre-lysed samples were extracted on the Roche MagNA Pure extraction system (Roche Diagnostics Limited, West Sussex, UK) according to manufacturer's instructions.

Library preparation for the Illumina MiSeq amplicon sequencing

Sample processing and library generation was performed as previously described by Lundberg et al. (173) Briefly, the library generation was as follows: PCR 1: Pre-amplification of 16S rRNA marker gene region is necessary for potentially low biomass

template in order to carry enough tagged amplicon through to the final indexing-amplification steps. Perform PCR using ~200 ng of gDNA from each sample. Using non-modified primers targeting positions 515F and 806R within the V4 region of the 16S rRNA marker gene prepare a mastermix solution [5µl 5x Phusion Hifi Buffer, 0.5µl (10 mM) dNTP, 1 µl (10 µM) V4 primer mix; 0.25 µl Phusion HS II polymerase and make to 25 µl per reaction using DEPC water] and amplify using the following condition: 98°C for 30 seconds (x1) 98°C for 10 seconds + 52°C for 30 seconds + 72°C for 20 seconds (10 cycles) 72°C for 5 minutes hold at 4°C for . Next clean-up the PCR products from PCR 1 using AxyPrep Mag PCR Clean-up kit as follows; vortex magnetic beads well before use to resuspend any magnetic beads that may have settled. Aliquot 15 µl of Axygen beads to 10 µl of PCR product into a sterile 96 well plate. Mix well by repeated pipetting and incubate at RT for 5 minutes. Place the reaction plate onto the IMAG magnetic separation device and wait until the liquid turns clear. Remove the clear liquid from the plate and discard. Next add 180 µl of 70% EtOH to each well of the reaction plate and incubate for 30 seconds at RT. Remove the 70% EtOH from each well and discard. Repeat the previous step once. Air dry the beads at RT for no more than 5 minutes and be careful not to over dry the magnetic beads as this will cause the beads to crack and lead to decreased elution efficiency. Ensure that all the ethanol has been removed from each well. Add 11 µl of molecular grade H2O to each well. Removereaction plate from the IMAG separation device and mix well by gentle vortexing. Placethe reaction plate onto the IMAG separation device for 1 minute to separate the beads from the solution. Transfer 10 µl of the cleaned-up PCR product to a sterile 96 well plate for the next PCR

step. PCR 2: Reverse Tagging Step using the cleaned product from PCR 1 using equimolar mixture of the reverse frameshift (FS) primers 808R_f1, 808R_f2, 808R_f3, 808R_f4, 808R_f5, 808R_f6). Primers are combined into a working stock of 0.5 µM. Perform 1 cycle PCR using 10µl of product from PCR 1. Prepare a mastermix solution [5 µl 5x Phusion Hifi Buffer, 0.5 µl (10 mM) dNTP, 2 µl (0.5 µM, Reverse_MT_tag Primer mix); 0.25 µl Phusion HS II polymerase and 7.25 µl DEPCwater] and amplify using the following condition: 98°C for 60 seconds (x1) 98°C for 10

seconds + 50°C for 30 seconds + 72°C for 60 seconds (1 cycle) hold at 4°C for . Next clean-up the PCR products from PCR 1 using AxyPrep Mag PCR Clean-up kit as follows; vortex magnetic beads well before use to resuspend any magnetic beads that may have settled. Aliquot 15 µl of Axygen beads to 10 µl of PCR product into a sterile 96 well plate. Mix well by repeated pipetting and incubate at RT for 5 minutes. Place the reaction plate onto the IMAG magnetic separation device and wait until the liquid goes clear. Remove the clear liquid from the plate and discard. Next add 180µl of 70% EtOH to each well of the reaction plate and incubate for 30 seconds at RT. Remove the 70% EtOH from each well and discard. Repeat the previous step once. Air dry the beads at RT for no more than 5 minutes and be careful not to over dry the magnetic beads as this will cause the beads to crack and lead to decreased elution efficiency. Ensure that all the ethanol has been removed from each well. Add 11 µl of DECP water to eachwell. Remove reaction plate from the IMAG separation device and mix well by gentle vortexing. Place the reaction plate onto the IMAG separation device for 1 minute to separate the beads from the solution. Transfer 10 µl of the cleaned-up PCR product toa sterile 96 well plate for the next PCR step. PCR 3: Forward-Tagging Step using the cleaned product from PCR 2

using equimolar mixture of the forward frameshift (FS) primers 515F_f1, 515F _f2, 515F _f3, 515F _f4, 515F _f5, 515F _f6). Primers are combined into a working stock of 0.5 µM. Perform 1 cycle PCR using 10µl of product from PCR 2. Prepare a mastermix solution [5 µl 5x Phusion Hifi Buffer, 0.5 µl (10 mM) dNTP, 2 µl (0.5 µM, Reverse_MT_tag Primer mix); 0.25 µl Phusion HS II polymerase and

7.25 µl DEPC water] and amplify using the following condition: 98°C for 60 seconds (x1) 98°C for 10 seconds + 50°C for 30 seconds + 72°C for 60 seconds (1 cycle) hold at 4°C for . Next clean-up the PCR products from PCR 3 using AxyPrep Mag PCR Clean-up kit as follows; vortex magnetic beads well before use to resuspend any magnetic beads that may have settled. Aliquot 17.5 µl of Axygen beads to 10 µl of PCR product into a sterile 96 well plate. Mix well and incubate at RT for 5 minutes. Next place thereaction plate onto the IMAG magnetic separation device and wait until the liquid goes clear. Remove the clear liquid from the plate and discard. Add 180 µl of 70% EtOH to each well of the reaction plate and incubate for 30 seconds at RT. Remove the 70% EtOH from each well and discard. Repeat previous step once. Air dry the beads at RT for no more than 5 minutes, be careful not to over dry the magnetic beads as this will cause the beads to crack and lead to decreased elution efficiency. Ensure that all the ethanol has been removed from each well. Add 16 µl of DEPC water to each well and remove the reaction plate from the IMAG magnetic separation device and mix well to resuspend the magnetic beads. Next place the reaction plate onto the IMAG separationdevice for 1 minute to separate the beads from the solution. Transfer 15 µl of the cleaned-up PCR product to a sterile 96 well plate for the next PCR step. PCR 4:Nextera-Adapter/Indexing Amplification step by performing a 34 cycle PCR, targeting the V4 region of the 16S rRNA marker

gene, using 15 µl of the cleaned reverse and forward tagged product from step PCR 3. Each reaction will have the same forward primers and a unique reverse primer which acts as the index (barcode) for each sample. The forward and reverse primers are typically diluted to a working stock of 5 µM and can be added separately to each reaction (the forward primer is universal and could be added to any master-mixes instead), or the forward primer can be added to each reverse primer in a working stock in a plate for further use. Prepare a mastermix solution [10 µl 5x Phusion Hifi Buffer, 1µl (10 mM) dNTP, 2.5 µl forward primer (SEQ_V4_F; AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATGTG); 2.5µl reverse primer (INDEX_R_bc1 to bc96; CAAGCAGAAGACGGCATACGAGAT XXXXXXXX GTGACTGGAGTTCAGACGTGTGCTC); 0.5 µl Phusion HS II polymerase and 7.25 µl DEPC water] and amplify using the following condition: 98°C for 30 seconds (x1); 98°C for 10 seconds + 63°C for 30 seconds + 72°C for 30 seconds (34 cycle) hold at 4°C for . Next run 5 µl of each reaction on a 1% agarose gel to visually confirm presence of products (~453bp). Clean the PCR products from step PCR 4 with AxyPrep Mag PCR Clean-up kit as follows; vortex magnetic beads well before use to resuspend any magnetic beads that may have settled. Aliquot 35 µl of Axygen beads and the entire PCR product into a sterile 96 well plate and mix well and incubate at RT for 5 minutes. Next place the reaction plate onto the IMAG magnetic separation device and wait until the liquid goes clear. Remove the clear liquid from the plate and discard. Add 180 µl of 70% EtOH to each well of the reaction plate and incubate for 30 seconds at RT. Remove the 70% EtOH from each well and discard. Repeat the previous step once. Air dry the beads at

R°T for no more than 5 minutes, avoiding to not over drying the magnetic beads as this will cause the beads to crack and lead to decreased elution efficiency. Next add 50 µl of DEPC water to each well and remove reaction plate from the IMAG magnetic separation device and mix well. Place the reaction plate back onto the IMAG magnetic separation device for 1 minute to separate the beads from the solution. Transfer all of the cleaned-up PCR product to a sterile 96 well plate. Next quantify products using Quant-iT™ PicoGreen® dsDNA Assay kit (Life Technologies, Paisley, UK) in a 96 well plate using 2 µl of cleaned product. Pool equimolar amounts from each sample adding no more than 20 µl of each reaction to the final pool. Typically, only reactions that failed will need to be added at this volume (the pool will not be equimolar for them). Gel purify the pool by running it on a 1% agarose gel and gel extracting the correct size band (~453bp) using the QIAEX II kit (Qiagen, Manchester, UK) according to manufactures instructions, removing as much of the excess agarose gel as possible. The final sample pool was quantified in triplicate using the Quant-iT™ PicoGreen® dsDNA Assay kit (Life Technologies, Paisley, UK) and the concentration converted to nM (minimum 4 nM required). Samples were stored at -20ºC/-80ºC until submission for Illumina MiSeq sequencing.

Molecular detection - Illumina MiSeq data processing

Paired-end Illumina MiSeq sequences were processed using QIIME (Quantitative Insights into Microbial Ecology; version 1.8.0), by joining the corresponding paired- end reads, removing the Illumina adapters and barcode sequences (173, 176). We removed

sequences with length less than 200 and longer than 400 nucleotides, as well as sequences with an average quality score (Phred score) of <Q30. Sequences lacking an exact match to a 5' primer were also removed from the dataset along with sequences that contained any mismatches in the barcode sequence. Following this initial processing step, we removed potential chimeric sequences from downstream processing through the implementation of Chimera Slayer (177). Sequences were then clustered into their representative OTUs based on the 97% sequence identity using the UCLUST algorithm (178), aligned against full length 16S rRNA marker gene sequences from the Greengenes reference alignment (version 13.8) by PyNAST and assigned their taxonomic identities according to the Ribosomal Database Project Classifier Tool (v 2.2) using an open reference OTU picking as implemented within QIIME (176, 179). Anumber of taxa were detected in the background of the negative control; however, thiscommunity lacked a strong dominance by any single taxon. Furthermore, singletons (i.e. taxa represented by a single read over all the samples) and OTUs representing potential human sequences, Archaea, Cyanobacteria, unassigned OTUs and those found in the background of the negative control were filtered out and treated as contaminating sequences prior to all downstream analysis. The resulting dataset was then converted to a final quality filtered OTU table and presented either normalised absolute counts or relative abundance.

Intra-sample similarities/differences were assessed through sample richness (number of counted taxa) and diversity (Shannon Wiener Index).

Blood samples

Blood samples were drawn into bottles containing sodium citrate at baseline and on a three-monthly basis after commencement of treatment. Blood samples were diluted 1:1 with PBS and inverted in a 15 ml falcon. 3 ml of Ficoll was pipetted into a centrifugetube. The diluted blood PBS mixture was layered gently onto the top of the Ficoll. The falcon was then centrifuged at 400g for 10 minutes at 18°C (acceleration 1, deceleration 1). After centrifuging the upper layer containing the plasma was pipetted into 2 ml Eppendorf's and frozen immediately at -80°C (Figure 3.3).

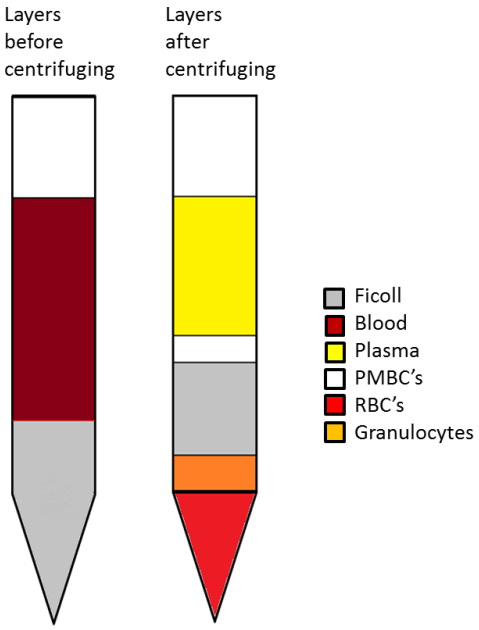


Figure 3.3 Ficoll banding procedure

***Anti-Pseudomonas aeruginosa* antibody measurement in blood samples**

Assay principle

Pseudomonas aeruginosa infection provokes a rapid production of antibodies to a large number of *Pseudomonas aeruginosa* antigens. Mediagnost antibody test uses three *P. aeruginosa* antigens, which are highly immunogenic and present in different parts from nearly all *P. aeruginosa* strains. Depending on the *Pseudomonas aeruginosa* species and the immune reaction, antibodies can be detected against a single, two or even all three antigens simultaneously. A sample is regarded as sero-positive when antibodies against one or more or the antigens can be detected.

The Mediagnost anti-*Pseudomonas aeruginosa* IgG anti-*Pseudomonas aeruginosa* IgG EIA, E15 is a sandwich enzyme immunoassay. Serum or plasma samples are diluted and added to the wells of a microtiter plate, which have been previously coated with the *Pseudomonas aeruginosa* antigens alkaline protease, elastase or exotoxin A. Specific antibodies in the sample bind to the antigens present during an incubation of 2 hours at 37°C. After washing, the conjugate (anti-human IgG peroxidase-labelled immunoglobulin) is added and incubated again for 2 hours at 37°C. After a final washing step, substrate is added and further incubated for 30 minutes at room temperature. The reaction is terminated on addition of stop solution accompanied by achange from blue to yellow. The absorbance of the coloured reaction product is measure on a microtiter plate reader. The colour intensity of the reaction corresponds to the concentration of the antibodies in the sample.

Assay procedure

All determinations (controls and samples) should be assayed in duplicate. When performing the assay, the positive control, negative control, control serum and the samples should be pipetted as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, the diluted conjugate concentrate KK as well as the following Substrate Solution S should be added to the plate in the same order and in the same time interval as the samples. Stop solution SL should be added to the plate in the same order as the Substrate Solution.

1. Pipette in position A1/2 100µl Negative control NK each
2. Pipette in position B1/2 100µl Positive control PK each
3. Pipette in position C1/2 100µl Control serum KS each
4. Pipette 100µl each of the diluted samples (generally 1:1000 diluted in dilution buffer VP) in the rest of the wells according to requirements.
5. Cover the wells with the sealing tape and incubate the plate for 2 hours at 37°C.
6. After incubation aspirate the contents of the wells and wash the wells 3 times with 300 µl of washing buffer. Empty the wells thoroughly.
7. Following the last washing step, pipette 100µl of the 1:100 diluted conjugate concentrate KK in each well.
8. Cover the wells with the sealing tape and incubate the plates for 2 hours at 37°C.
9. After incubation wash the wells 3 times with washing buffer as described in step 6.
10. Pipette 100µl of the TMB-substrate solution S in each well.
11. Incubate the plates for 30 minutes in the dark at room temperature.

12. After incubation pipette 100µl of stop solution SL in each well. Measure the absorbance within minutes at 450 nm.

Calculation of results

Calculations should be performed for each antigen (each plate) separately. Calculate the average of all multiple values. For the evaluation of the assay it is preconditioned that the absorbance values of the negative controls should be below 0.25. The difference between the extinctions of negative controls and the respective positive controls must be at least 0.6.

Qualitative calculation:

The negative control average is subtracted from the controls and samples to obtain absolute values.

Cut-off calculation:

The cut-off is 20% of the positive control average. This value corresponds to a titre of 1:500 of a 1:1000 diluted serum. 1:1000 diluted sera with extinction values less than the cut off are regarded as negative. Depending on the *Pseudomonas aeruginosa*species and the immune reaction antibodies can be detected against a single, two or even all three antigens simultaneously. A sample is regarded as sero-positive when it is positive for one or more of the antigens.

Semi-quantitative calculation

The measured extinction values of the individual serum dilutions are plotted onto a graph or calculated using an appropriate computer programme (Graphpad prism). The extinction values of the negative (NK) and positive (PK) controls are plotted on the y- axis of a linear plot. A so-called titre factor is in turn plotted on the x-axis, for NK = 1and PK = 2.5. For the quantification of the sera values a straight line is drawn through the NK and PK values and extended to a value of 3.5. The titre of the individual serumis determined by reading the titre factor of the measured extinction value through the NK-PK axis, which is multiplied by the serum dilution factor. Titre factors lower than 0.25 and higher than 3.5 (x-axis) are not taken into consideration in these calculations, except that the titre factors from a 1:1000 dilution below 0.5 are regarded as negative and titre factors greater than 3.5 are always positive and should be dilute further and retested for quantitative determination.

Categories:

The semi-quantitative detection of human sera using the Mediagnost anti- *Pseudomonas aeruginosa* IgG EIA, E15, is subdivided into the following categories (Table 3.1).

Table 3.1 *Anti-Pseudomonas* antibody interpretation

Titre	**Interpretation**
<1:500	Negative
1:500 to 1:1250	Borderline
>1:1250	Positive
>1:10,000	Chronically positive

Statistical Analysis

Data was analysed using SPSS version 22·0 and STATA. A mean post follow-up value was calculated for each parameter for each patient (mean of available 3, 6, 9- and 12-month values) and this post value was compared to the value before commencing ivacaftor. Paired sample t test was used to compare change in normally distributed data and paired sample Wilcoxon signed rank test was used to evaluate median changein non-normally distributed data. Repeated measures ANOVA with a Bonferroni correction for multiple comparisons were used to compare change in chest CT score. Pearson correlation and Spearman Rank correlation coefficients were used to evaluate correlation between clinical parameters and Chest CT scores depending on whether they were normally distributed or not.

For the lung microbiota assessment of the data demonstrated that for most of the included variables the data did not conform to normal normality (Shapiro-Wilks normality test; $p \leq 0.05$). Hence, nonparametric analyses were performed, which included the Mann-Whitney U test, Wilcoxon signed-rank test, Spearman's (ρ) ranked correlation and Pearson (r) correlation test for count based and clinically relevant data where appropriate. The analysis of microbial community based (extended culture and molecular based data) and clinical data was conducted in the R environment (http://www.r-project.org). *P <0.05* was accepted as statistically significant.

Results

A significant 109 metre mean increase in modified shuttle walk test was observed after commencement of ivacaftor treatment (*p=0·001*) (Figures 3.4).

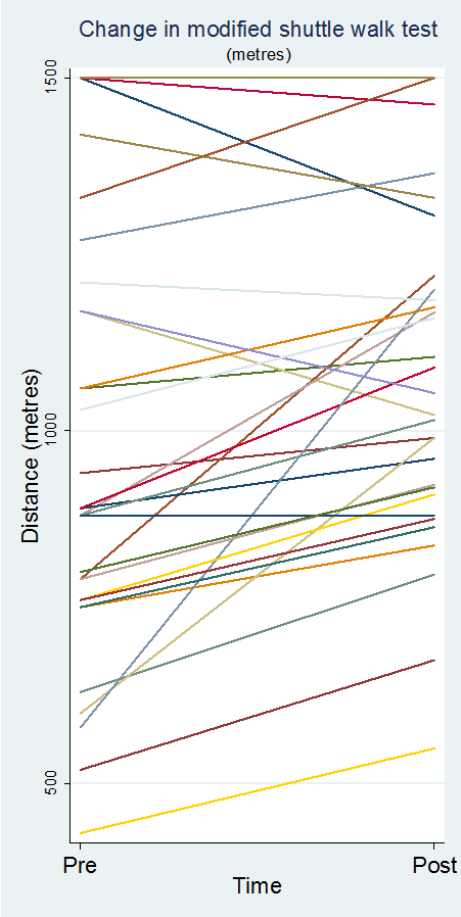


Figure 3.4 Modified shuttle walk test before and after ivacaftor (n=33)

Radiology

Eighteen adults had a low dose chest CT performed before commencing ivacaftor and after 3, 6 and 12 months of treatment. In the adults who had CT imaging performed a 12% mean increase in FEV_1 % predicted (*p<0·01*), 58 mmol/l mean reduction in sweat chloride (*p < 0·01*) and 1·6 Kg/m^2 mean increase in BMI were observed after commencement of ivacaftor. Table 3.2 summarises the CT findings. Repeat measures ANOVA demonstrated significant mean reductions in Total Bhalla Score (*p < 0·01*), peri-bronchial thickening (*p = 0·035*), and extent of mucus plugging (*p < 0·01*) with treatment. There was no statistically significant change in the other aspects of Bhalla score including the severity and extent of bronchiectasis, number of bullae, emphysema, presence of sacculation or abscesses and the generations of bronchial divisions involved in bronchiectasis and plugging (Table 3.2). Post hoc testing using a Bonferroni correction for multiple comparisons demonstrated significant improvement from baseline in total Bhalla score (*p < 0·01*) and mucus plugging (*p < 0·01*) after 3, 6 and 12 months.

Table 3.2 Mean Chest CT Bhalla Score before and after ivacaftor (n=18)					
Bhalla score (Range)	Baseline Mean score (SD) N = 18	3 months Mean score (SD) N = 18	6 months Mean score (SD) N = 18	12 months Mean score (SD) N= 18	*P value*
Total Bhalla score (0- 25)	12.56 (4.2)	10.94 (3.6)	10.22 (3.4)	10.33 (3.4)	*<0.01*
Peri-bronchial thickening (0-3)	1.11 (0.8)	0.89 (0.7)	0.83 (0.7)	0.72 (0.6)	*0.035*
Severity of bronchiectasis (0-3)	2.06 (0.9)	2.11 (0.9)	2.06 (0.9)	2.06 (0.9)	*0.33*
Extent of bronchiectasis (0-3)	2.83 (0.4)	2.83 (0.4)	2.78 (0.4)	2.83 (0.4)	*0.33*
Extent of mucus plugging (0-3)	1.83 (1)	1.0 (0.9)	0.67 (0.69)	0.67 (0.84)	*<0.01*
Sacculation or abscesses (0-3)	0 (0)	0 (0)	0 (0)	0 (0)	*1*
Generations of bronchial divisions involved (0-3)	2.44 (0.7)	2.4 (0.8)	2.44 (0.7)	2.4 (0.8)	*0.7*
Number of bullae (0-3)	0.67 (1.1)	0.5 (0.9)	0.5 (0.9)	0.44 (0.9)	*0.3*
Emphysema (0-2)	1.17 (0.7)	0.94 (0.7)	0.7 (0.6)	1.0 (0.6)	*0.15*
Collapse/ consolidation (0-2)	0.44 (0.6)	0.28 (0.6)	0.22 (0.4)	0.22 (0.4)	*0.1*

Figure 3.5 and 3.6 illustrate representative chest CT before and after treatment.

Figure 3.5 CT Thorax before (A) and after (B) ivacaftor

The images demonstrate an interval resolution of right middle lobe medial segmental consolidation and collapse and reduction in the degree of peribronchial wall thickening and foci of mucous plugging in the left and right lower lobes. Persistent residual mucous plugging in the lingula anteriorly.

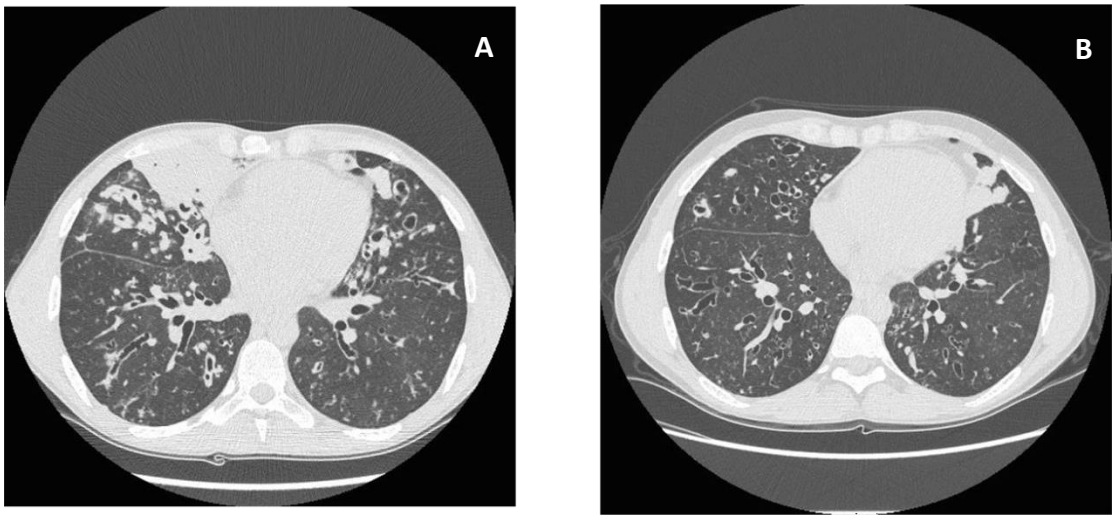
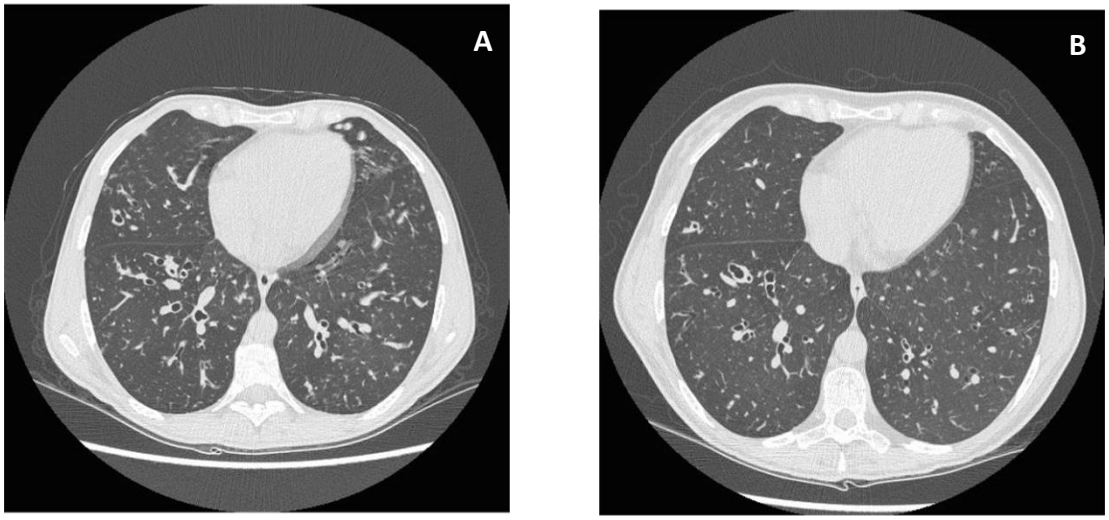


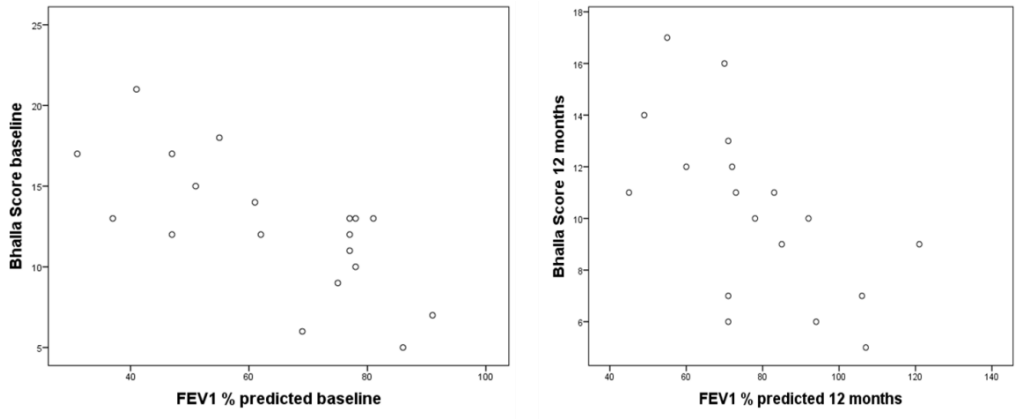
Figure 3.6 Chest CT before (A) and after (B) after ivacaftor

The image demonstrates an interval reduction in the degree of peri-bronchial wall thickening, mucous plugging and tree in bud opacification, with the degree of bronchiectasis remaining unchanged.



A significant inverse correlation was observed between FEV_1 % predicted and total Bhalla Score at baseline (*r = -0·65, p=0·002*) and after 12 months (*r = – 0·69, p= 0·002*)of therapy, reflecting that those with lower FEV_1 had worse disease on chest CT as evidence by a higher total Bhalla score (Figure 3.7). There was no correlation between the magnitude of change in FEV_1 and the magnitude of change in total Bhalla score after treatment.

Figure 3.7 Correlation between FEV_1 % predicted and CT Bhalla Score at baseline and after 12 months of ivacaftor (n=18)



Extended bacterial culture

Bacteria were cultured in high numbers from all sputum samples collected PRE- (up to 2.08 x 10^8 CFU/g of sputum) and POST-treatment (up to 1.25 x 10^8 CFU/g of sputum) with ivacaftor. There was no significant difference in the total bacterial density cultured from PRE- (1.32 x 10^7 [3.00 x 10^4 to 8.60 x 10^7] CFU/g of sputum; median

[range]) or POST-treatment (8.52 x 10^6 [3.60 x 10^5 to 5.60 x 10^7] CFU/g of sputum; median [range]) samples (Figure 3.8A). Aerobic bacteria were cultured in high abundance from all PRE- (up to 2.07 x 10^8 CFU/g of sputum) and POST-treatment (up to 1.08 x 10^8 CFU/g of sputum) samples. There was no significant difference in the median values of total aerobic bacterial density from PRE- (1.23 x 10^7 [3.00 x 10^4 to 8.60 x 10^7] CFU/g of sputum; median [range]) versus POST-treatment (7.49 x 10^6 [3.60 x 10^5 to 4.79 x 10^7 CFU/g of sputum; median [range]) samples (Figure 3.8B). Anaerobic bacteria were cultured from 6/10 PRE- (up to 1.40 x 10^6 CFU/g of sputum) and from all 10 POST-treatment samples (up to 1.71 x 10^7 CFU/g of sputum). The total anaerobic bacterial density Pre-treatment (4.31 x 10^4 [0.0 to 2.39 x10^6] CFU/g of sputum; median [range]) was significantly lower than POST-treatment (3.50 x 10^6 [9.26 x 10^3 to 2.96 x 10^7] CFU/g of sputum; median [range]) (*p = 0.024*, Figure 3.8C)

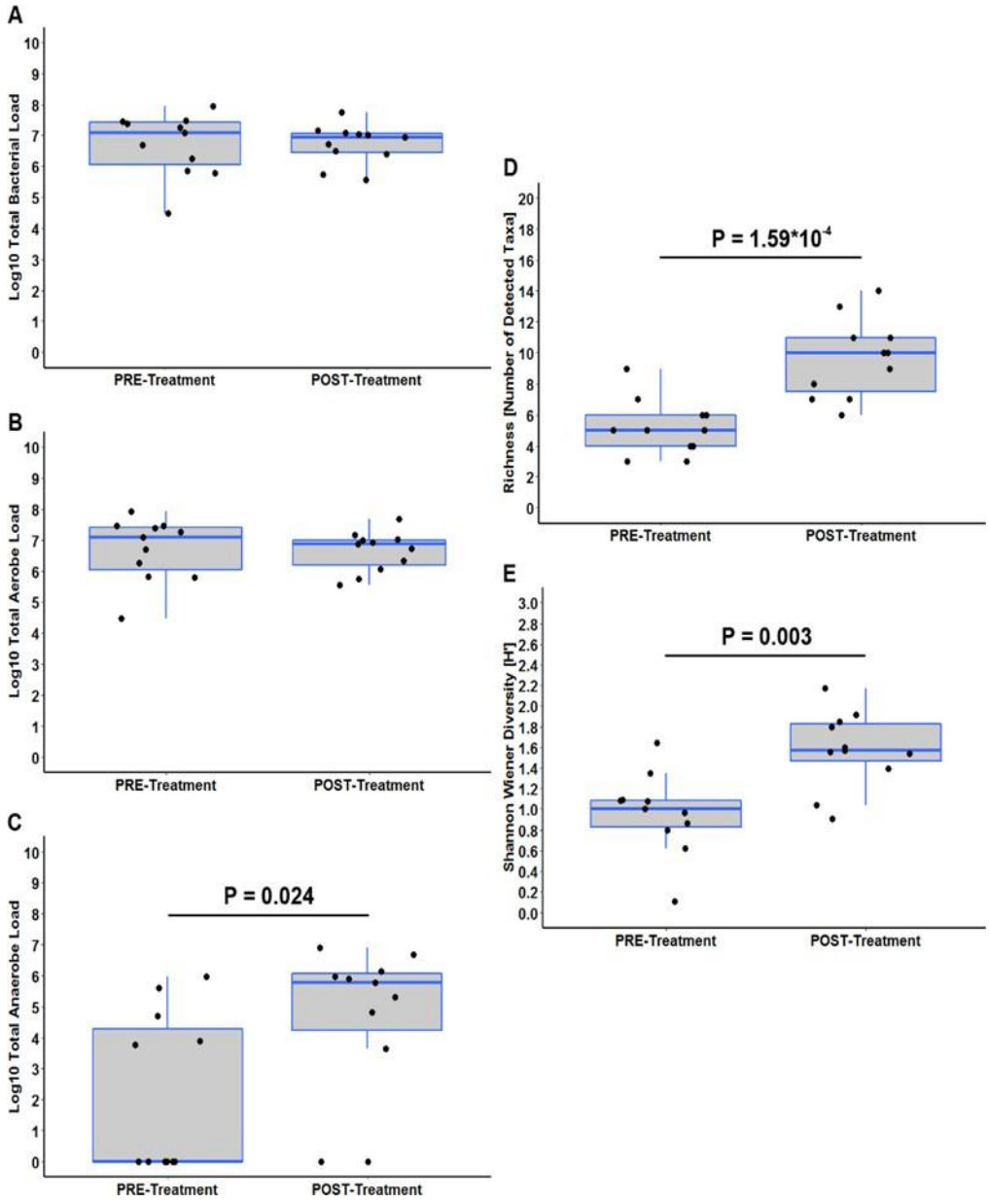


Figure 3.8 Comparison of viable counts/g of sputum and ecological parameters by extended-bacterial culture in sputum samples collected from PWCF PRE- and POST-treatment with ivacaftor. (A) total bacterial load (B) total load for aerobic bacteria (C) total bacterial load for anaerobic bacteria (D) taxonomic richness and (E) community diversity (Shannon Wiener Index [H']). *P<0.05* denotes statistical significance.

In total, we cultured 54 different bacterial taxa with no single taxa demonstrating significant difference PRE- and POST-treatment. All 6 patients who were *P. aeruginosa* positive by extended-culture pre-treatment were also *P. aeruginosa* positive POST-treatment with no significant difference in density. Significant differences were detected between PRE- and POST-treatment samples for both taxonomic richness and community diversity ($p=1.590 \times 10^{-4}$ and $p=0.003$, respectively) (Figure 3.8).

qPCR quantification of total bacterial and *P. aeruginosa* density

No significant difference in fold change was observed for either total bacterial 16S rRNA copy number [6.30×10^8 (PRE) and 4.14×10^8 (POST)] or *P. aeruginosa* density for oprL copy number [1.65×10^7 (PRE) and 1.79×10^7 (POST)] following ivacaftor therapy. We observed significant concurrence between *P. aeruginosa* TVC's (extended-culture) and oprL qPCR values for *P. aeruginosa* (*Pearson* product-moment *correlation* coefficient r = 0.56; $R^2 = 0.301$; $p = 0.007$; Bonferroni adjusted for multiple comparisons) (Figure 3.9). Furthermore, there was excellent agreement between the relative abundance of *Pseudomonas spp*. from Illumina MiSeq 16S rRNA marker-gene sequencing and oprL qPCR values for *P. aeruginosa* (*Pearson* product-moment correlation coefficient r=0.88; R*2*=0.775; $p=3.91^{*10-10}$; Bonferroni adjusted for multiple comparisons) (Figure 3.9).

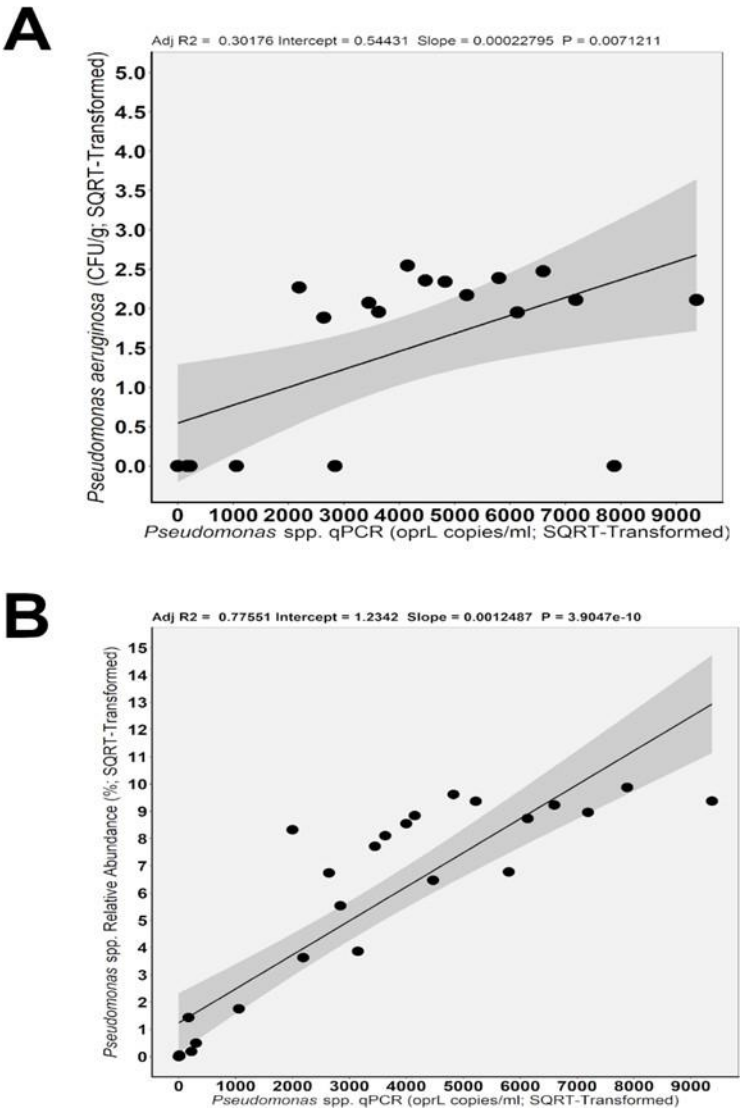


Figure 3.9. Pearson's correlation coefficient demonstrating the relationship between (A) the total viable counts (CFU/g sputum) of *Pseudomonas aeruginosa* (from culture-dependent analysis) with *P. aeruginosa opr*L copy number (copies/ml) as quantified by qPCR. Pearson's correlation coefficient r = 0.556 (R^2 = 0.301; *p = 0.007*) and (B) between the relative abundance (%) of *Pseudomonas* spp. (from Illumina MiSeq 16S rRNA marker-gene sequencing) with *P. aeruginosa opr*L copy number (copies/ml) as quantified by qPCR. Pearson's correlation coefficient r = 0.885 (R^2 = 0.776; $p = 3.91*10^{-10}$).

Illumina MiSeq sequencing

Following quality filtering and pre-processing of the paired-end sequence reads, each sample was normalised to 58,291 reads. In total, we detected 179 different taxa in the paired samples (PRE-ivacaftor [n=123]; POST-ivacaftor treatment [n=160]). Furthermore, a limited number of taxa (26/179) accounted for 99% of the total sequence read number in our cohort. At the phylum level, the mean relative abundance of *Proteobacteria* was reduced by 22% POST-treatment, while mean relative abundance of *Firmicutes, Bacteriodetes, Actinobacteria* and *Fusobacteria* increased by 12%, 13%, 57% and 500%, respectively (Figure 3.10A). At genera level, there was a significant change in community composition following treatment in samples from 9/14 PWCF, with only two of these individuals receiving IV antibiotics in the previous 8 weeks. In 5/14 PWCF, community composition remained relatively stable following treatment with only marginal changes in the relative abundance of thedominant genera (Figure 3.10B). None of the 5/14 received IV antibiotics in the previous 8 weeks. Comparison of taxonomic richness demonstrated a significant increase in observed taxa richness POST-treatment (Figure 3.10A; *p=0.031*, Mann-Whitney test). Community diversity (Shannon–Wiener index [H']), although trending towards a higher diversity in the POST-treatment samples, did not show a significant difference between visits (Figure 3.10B, *p=0.069*; Mann-Whitney test). Community evenness (eH/S) anddominance (D) were similar PRE- and POST-treatment (*p=0.085* and *p=0.094*, respectively; Mann-Whitney test).

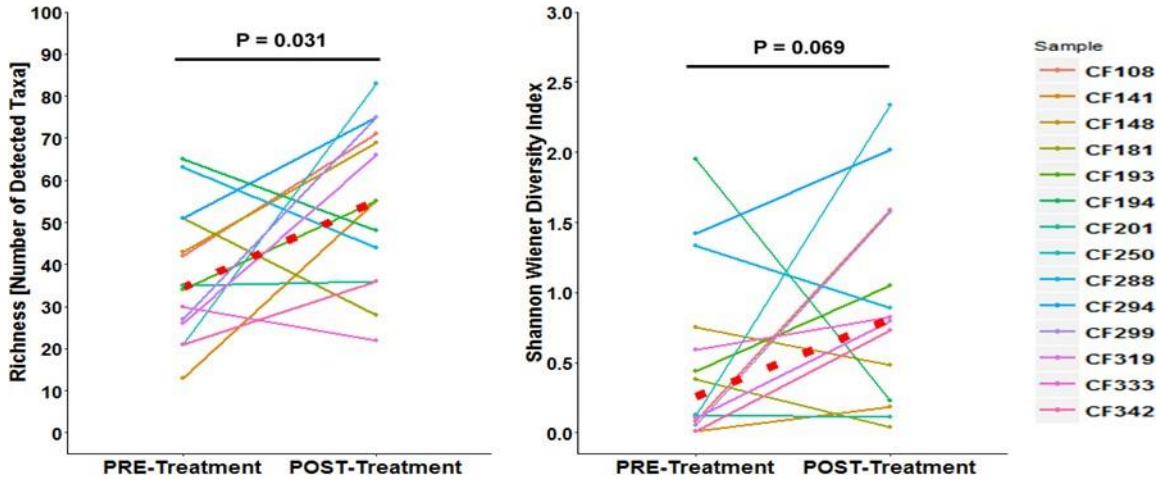


Figure 3.10 Line plot displaying the direction of change following treatment with ivacaftor for molecular based analysis. (A) taxonomic richness (B) community diversity (Shannon Wiener Index [H']). Red dotted line demonstrates the change in mean values. *P<0.05* denotes statistical significance.

We observed a high degree of variation in the microbiota between patients (Figure 3.10B). Inter- similarities/differences, based on β-diversity computed with the Bray-Curtis dissimilarity measures, demonstrated a significant difference between individual patients (R^2 = 0.70; *p=0.006*; sample ADONIS; 9999 permutations). In contrast, analysis between PRE- and POST-treatment samples was not shown to be statistically significant (R^2 = 0.01; *p=0.653*; PERMANOVA; 999 permutations) (Figure 3.11).

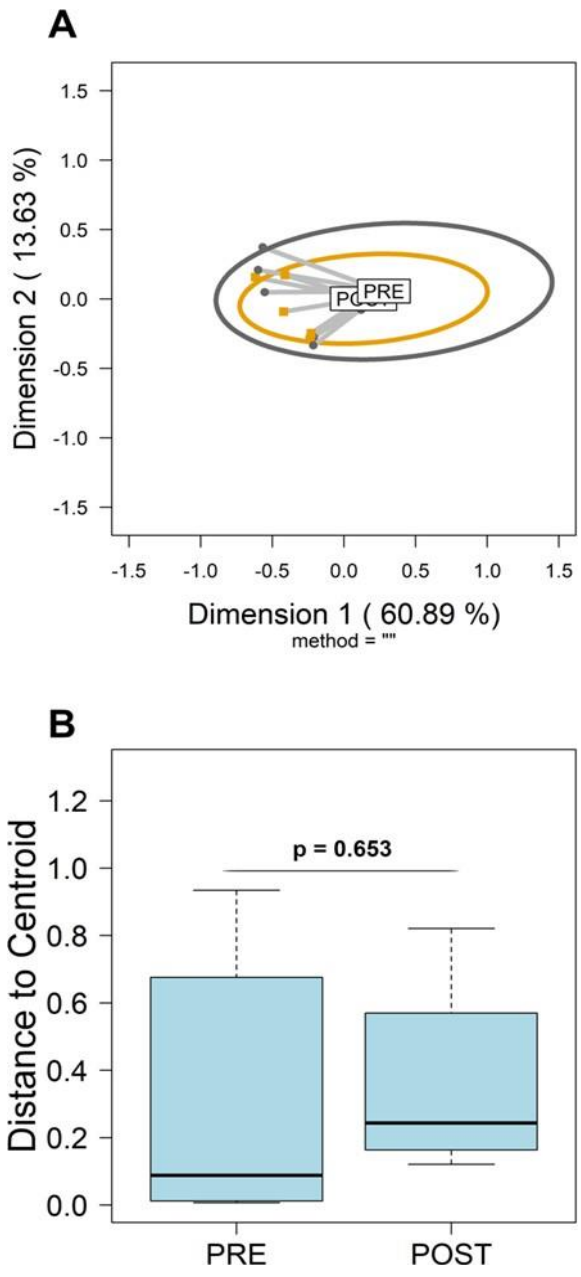


Figure 3.11. Differences in the compositional variance calculated using the Bray-Curtis distance measure and the "betadisper" function from the vegan package (2.4-6) in R, followed by significance testing using a permutation test. The permutation tests assess significant differences of median distance to centroid. PRE- and POST-ivacaftor visits: (A) distances to the centroids on the first two Principle Co-ordinates Analysis (PCoA) axes (90% confidence interval) and (B) distribution of variance. *P*<0.05 denotes statistical significance with 999 permutations.

Communities dominated by a high relative abundance of members of *Pseudomonas spp*. were associated with lower taxonomic richness and diversity (Figure 3.12) while communities with relatively higher abundance of members of *Streptococcus spp*. were associated with both higher taxonomic richness and diversity (Figure 3.12). There was a general change in the direction of community composition following ivacaftor treatment from communities dominated by *Pseudomonas spp., Stenotrophomonas spp.* (classified as a "family of unclassified Xanthomonadaceae") and *Staphylococcus spp*. Pre-treatment towards communities with a higher relative abundance of *Streptococcus spp.* POST-treatment (Figure 3.12)

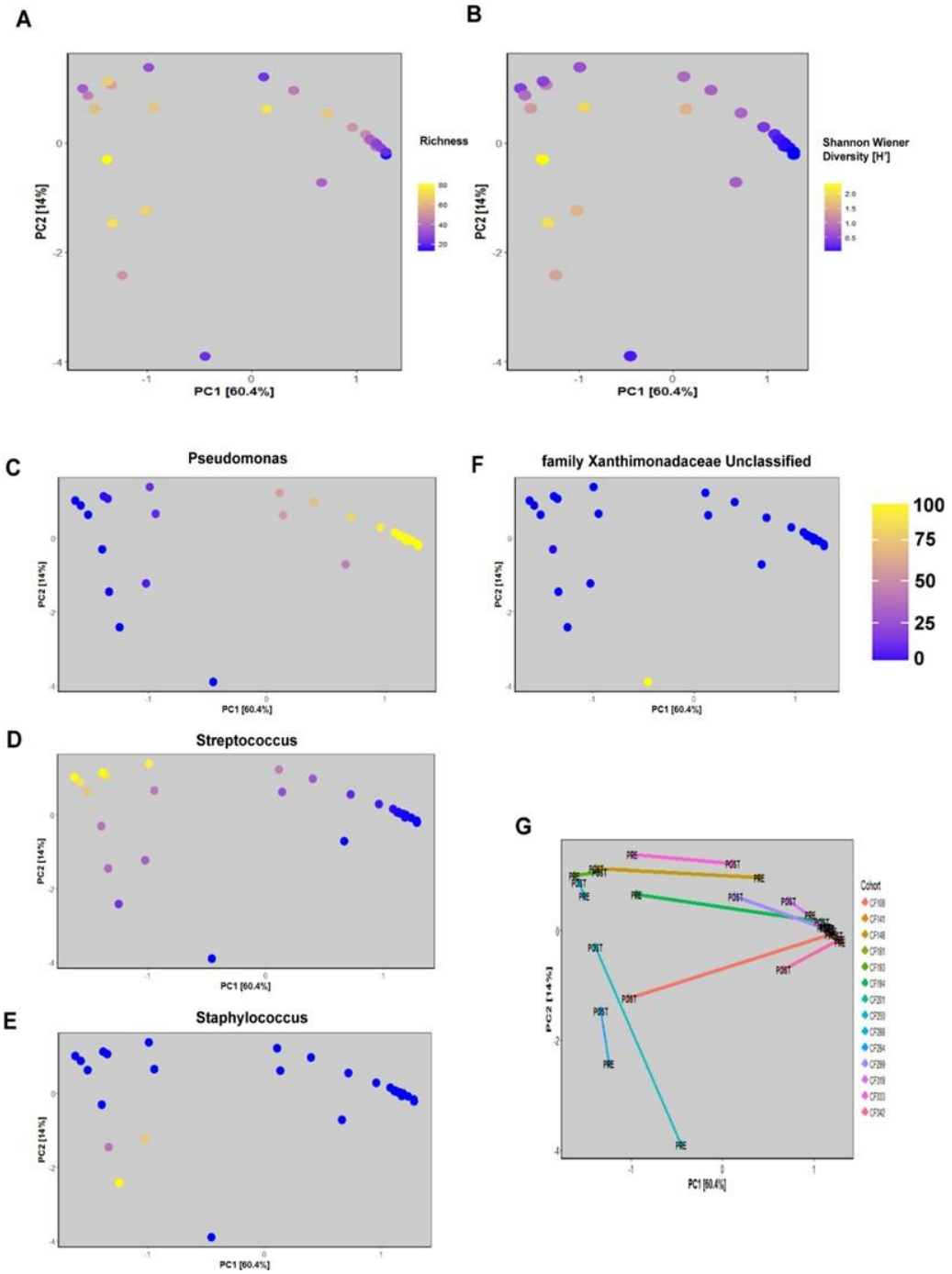


Figure 3.12. Principal Component Analysis (PCA) for the two main ecological community estimators: (A) Richness and (B) Shannon Wiener Diversity [H']. PCA analysis for the four main genera based on relative abundance (% normalised counts): (C) *Pseudomonas* spp., (D) *Streptococcus* spp., (E) *Staphylococcus* spp. and (F) unclassified *Xanthomonadaceae. (G) Direction of community changes from PRE- to POST-ivacaftor treatment.* The plot of the PCA axis 1 accounts for 60.4% of explained variation and the PCA axis 2 accounts for 14.0% of the variation explained.

Anti-*Pseudomonas aeruginosa* antibody

Anti-*Pseudomonas aeruginosa* antibodies were measured in blood samples from 21 patients with CF before and after commencing ivacaftor. A 10% mean increase in FEV_1 % predicted ivacaftor (*p <0.001*) and 75% reduction in pulmonary exacerbations requiring intravenous antibiotics (*p = 0.036*) was observed in this sub-group in the year after ivacaftor. There was no significant change in anti-*Pseudomonas aeruginosa* antibody levels measured in relative ELISA units after ivacaftor for the protease (*p = 0.79*), Exotoxin A (*p = 0.87*) or Elastase (*p = 0.57*) assay (Figure 3.13).

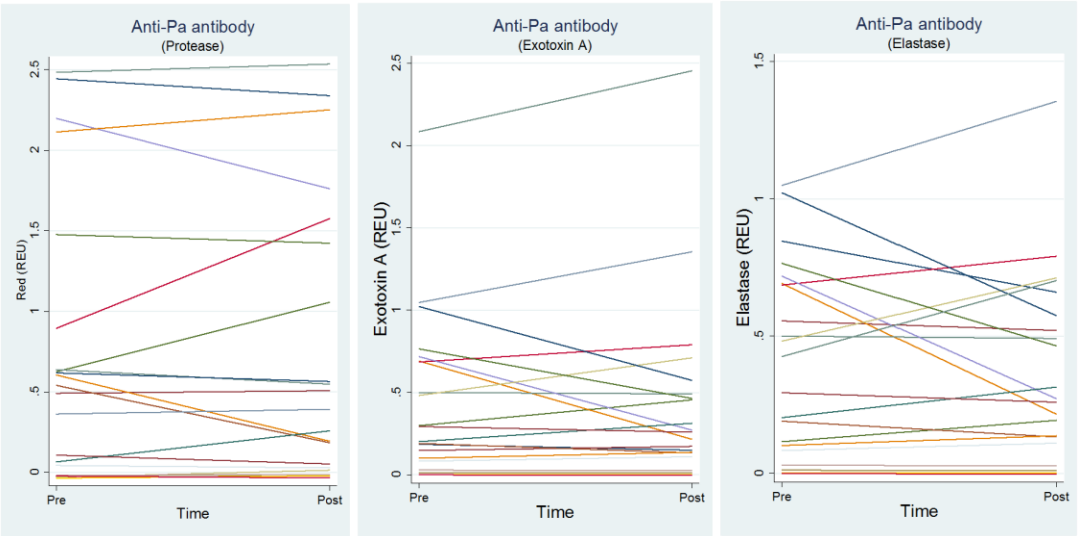
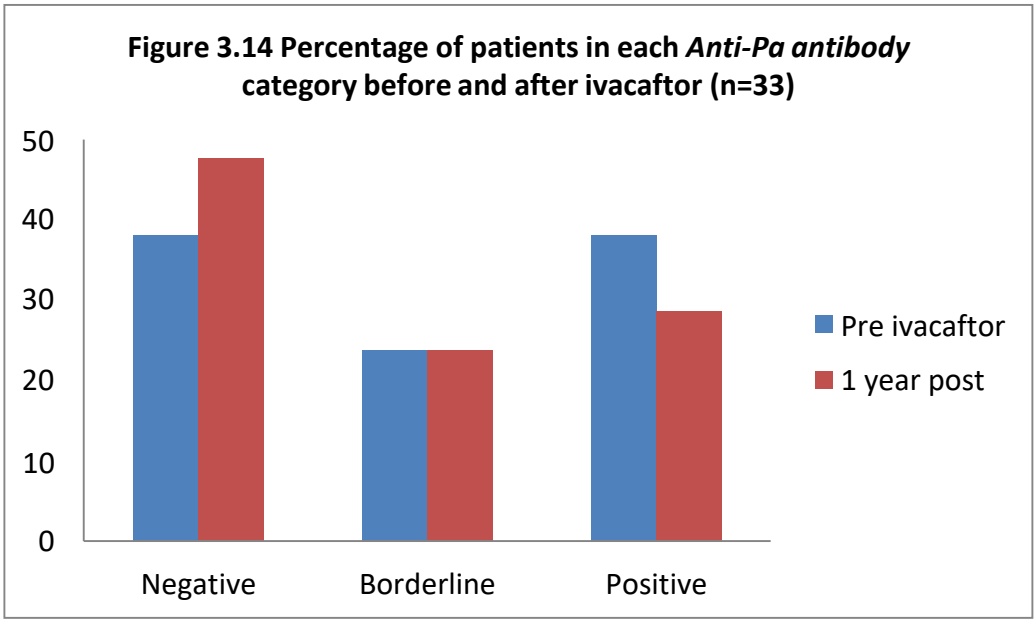


Figure 3.13 Anti-*Pa antibody* titres before and after ivacaftor (n=33)

There was no significant change in anti-*Pseudomonas aeruginosa* antibody category after ivacaftor (*p = 0.6*) (Figure 3.14).



Discussion

There was a significant improvement in modified shuttle walk test after ivacaftor. An advantage of this measurement is that it is suitable for use in all age groups and across the full spectrum of lung disease severity (132, 170). Importantly, measurement of exercise tolerance and capacity appears to be a useful tool in evaluating morbidity, prognosis and transplant suitability in people with CF (133, 180, 181). Previous work has demonstrated the validity of the modified shuttle walk test as an objective markersof exercise ability in patients with CF (182). Modified shuttle walk test distance has been shown to improve after hospitalisation and treatment with intravenous antibiotics for an exacerbation of CF (183). As a result, there is reason to hypothesise that modified shuttle walk test might be a useful and simple means of evaluating treatment response in patients treated with CFTR modulators. A limitation of this assessment modality is the ceiling effect, given that the maximum distance is 1500

metres. The improvement me observed in exercise after ivacaftor is in keeping with an Australian study which demonstrated improved exercise capacity after ivacaftor (184). This is the first study to use ultra-low-dose chest CT scanning (mean dose, 0.08 mSv per scan) to serially examine the ivacaftor response over a 1-year period, allowing an assessment of the key mechanisms underlying clinical response and assessing its utility in a clinical setting to monitor CFTR modulatory therapy response. Significant improvements in ultra-low-dose chest CT scans were observed early, after 3 months of treatment, with further improvements noted after 1 year of therapy. The greatest improvements on chest CT images were in the extent of mucous plugging. This is consistent with in vitro studies in which ivacaftor enhanced airway surface liquid and ciliary beat frequency (17, 32). The limitation of using FEV_1 to assess treatment response, in particular in patients with relatively preserved lung function, is well recognized (107). Accordingly, we suggest that ultra-low-dose chest CT scanning may represent a biomarker of early and sustained treatment response post-CFTR modulation. It may be a useful potential new outcome measure in clinical care, with broad application across CF sites. The growing awareness that patients with CF are at increased risk of certain neoplasms supports using low-dose CT scanning to reduce this risk (185, 186). Importantly there is evidence to suggest an increased risk of malignancy of 1 in 1000 in people who are exposed to ≥ 10mSv of cumulative effective radiation (187). Follow-up data over a 17-year period and comprising 2,240-person years of follow up in a cohort of patients with CF demonstrated increasing radiation exposure over time and that 27% of patients had a cumulative effective radiation exposure

above this threshold (186). Thus, utilising a low radiation exposure would be important. There was no statistically significant change in bronchiectasis on chest CT scans after ivacaftor therapy in contrast to a previous smaller study that included adults and children (77). Our study supports the finding that in established lung damage, bronchiectasis is not reversible with CFTR modulatory therapy. It remains to be established if treatment with ivacaftor will delay or prevent the development of bronchiectasis in those who start treatment early.

This is the first study to simultaneously use extended quantitative culture and culture-independent methods to assess changes in microbial community composition following ivacaftor treatment. Extended culture detected a high number of both aerobic and strict anaerobic bacteria prior to commencing and POST-ivacaftor treatment, with total anaerobic bacterial numbers, community richness and diversity significantly higher following treatment. Culture-independent analysis did not demonstrate a significant effect on airway microbial community composition following ivacaftor treatment, despite significant changes being observed within individual patients. In general, where such changes occurred, the shift was from communities with a higher proportion of *Pseudomonas spp*., *Stenotrophomonas spp*. and *Staphylococcus spp*. towards communities with a higher proportion of *Streptococcus spp*. Following treatment, community structures displayed an increase in taxonomic richness as well as a trend towards higher community diversity. Furthermore, we observed a significant inverse relationship between community richness and markers of inflammation in blood. This

shift is in *P. aeruginosa* the direction of a microbiota associated with less severe CF lung disease (188).

A key finding from the current study is that, despite significant improvements in many of the clinically relevant parameters, ivacaftor treatment did not result in eradicationof *P. aeruginosa* from the airways. This contrasts with the findings of Heltshe and colleagues who reported a significant reduction in *P. aeruginosa* culture positivity one year after initiation of ivacaftor therapy (189). However, the results of this earlier study may have been skewed by the inclusion of patients defined as having intermittent infection prior to initiation of ivacaftor therapy. Our findings are similar to those of Hisert et al. who followed PWCF longitudinally for up to 3 years and reported that noneof the 8 patients chronically colonised with *P. aeruginosa* eradicated their infecting strain (190). We did not demonstrate, by either culture or qPCR, a significant difference in *P. aeruginosa* density in PRE- and POST-treatment samples. In contrast, Hisert et al., who performed more frequent sample analysis, reported a significant reduction in *P. aeruginosa* density in the first week of treatment which was maintained for approximately 7 months (day 210). However, *P. aeruginosa* density increased in 6/7 patients over the remaining study period (190). As our POST-treatment samples were collected at either 9 or 12 months, it is likely that any initial reduction in *P. aeruginosa* density, if present, would have been reversed by 9 or 12 months and therefore not detected.

Of interest, Peleg and co-workers demonstrated that, although significant changes in microbial community composition occurred following ivacaftor treatment, they were primarily as a result of concurrent antibiotic exposure. PWCF that did not receive intravenous antibiotic treatment only demonstrated modest changes in their microbiota (191). However, only 2/14 PWCF in our study that displayed a significant shift in microbial community composition had received a course of intravenous antibiotics in the eight weeks preceding collection of the POST-treatment sample. Changes in the lung environment, such as modulation in pH levels of the airway surfaceliquid (ASL) can have a significant effect on its viscosity (192), stabilisation of mucin binding and local host defence mechanisms (193). This suggests that changes in the airway environment could play a significant role in shaping community composition as well as modulating the virulence potential of a number of the main pathogenic taxa as these were seldom eliminated from the community following ivacaftor treatment (194, 195).No direct association between individual bacterial taxa, community diversity or taxonomic richness and lung function were detected.

The current study has a number of limitations. As this is a single centre study, inferring the results to findings elsewhere may be difficult. However, the current study is the

largest single centre study reported to date. Importantly, we used both extended-culture and culture-independent methods to examine the effect of ivacaftor on bacterial communities present in sputum from PWCF in a region that shows the highestprevalence of the G551D-*CFTR* mutation in the world. Our study is also observational innature and did not include a matched control group of individuals that did not receive ivacaftor. However, the benefits of ivacaftor relative to placebo have been well demonstrated in existing trials (20, 21), and the observed clinical improvements in the current study are comparable to those previously reported.

Interestingly there was no significant change in anti-*Pseudomonas aeruginosa* antibody levels or status after ivacaftor. This does raise the possibility that the reduction in relative abundance of *Pseudomonas* after treatment may represent reduced sputum production and thus variable lung sampling. A case reported a patient previously chronically colonised with *Pseudomonas aeruginosa* who's sputum became culturenegative for 16 months after commencing ivacaftor (196). However, a subsequent sputum sample cultured the original infecting strain of P*seudomonas aeruginosa* using 16s RNA sequencing (196). This highlights the need for caution in interpreting patients *Pseudomonas* status and in de-escalating *anti-Pseudomonal* therapy in patients previously chronically colonised with *pseudomonas aeruginosa* who appear to the *Pseudomonas* free after ivacaftor.

Given the sustained improvements in chest CT images observed from 3 months after commencement of ivacaftor treatment (in particular, improved mucous plugging and

peribronchial thickening), we suggest that enhanced mucus clearance is a key mechanism underlying the increase in lung function and reduction in pulmonary exacerbations observed in patients treated with ivacaftor. This is consistent with in vitro studies in which ivacaftor resulted in improved airway surface liquid and ciliary beat frequency (32, 33).

Limitations

The limitations of this work are the same as the preceding chapter, its observational nature and the lack of a control group.

Conclusion

This study demonstrates sustained improvement after ivacaftor treatment across multiple novel modalities of assessment, including exercise testing, ultra-low-dose chest CT imaging, the lung microbiome. It suggests the potential utility of ultra-low- dose chest CT imaging as an approach for assessing treatment response. Variation in microbial community composition and the prevalence of pathogenic taxa were highly individual without an overall significant change in either following ivacaftor treatment. Changes in the airway environment and circulating inflammatory cytokine levels as a consequence of ivacaftor treatment, may result in an indirect effect on the local microbiota and modulate the virulence of pathogenic members of the community.

Chapter 4

The effect of CFTR modulation on extrapulmonary comorbidities

Introduction

The global burden of disease study 2013 demonstrated an increase in the number of people living with multiple comorbidities, with the aging of the global population (197). Thirty-two percent of adults aged 20–64 have 5 or more comorbidities and the number of comorbidities in an individual increased with age (197). Life expectancy is increasing with CF. A recent European CF Registry study highlighted that by the year 2025, there would be a 75% increase in adults with CF (198). These calculations were based on a cohort of patients who were CFTR modulatory therapy naive. CFTR modulation treatment offers a new personalised approach to CF care and given its systemic mode of action there is potential for modification of both pulmonary and non-pulmonary CFTR disease and outcome (199). Given the improved CF survival with traditional approaches, the potential of augmenting this by new CFTR modulation, whilst working in a clinical arena where morbidities in aging populations this chapter aims to evaluate the effect of CFTR modulation on extra-pulmonary comorbidities including circulating inflammatory mediators, and pancreatic exocrine and endocrine function.

Circulating inflammatory mediators in CF

The cytokine system plays a key role in a variety of inflammatory and infectious diseases (200, 201). Previous studies have demonstrated elevated levels of a variety of circulating blood cytokines including IL-6, IL-8, and TNF-α in PWCF compared to control cohorts (202-204). Elevated levels of neutrophil elastase and a range of other cytokines have been demonstrated in sputum and Broncho-alveolar lavage (BAL)

samples form people with CF (205). This excess inflammation begins in very early life in CF, with previous work demonstrating elevated levels of pro-inflammatory cytokines in BAL samples from infants with CF even in the absence of obvious bacterial lung infection (206). This increased level of circulating and lung cytokines suggest that there is a persistent excess inflammatory response in CF which likely contributes to ongoing lung damage. Furthermore, elevated levels of circulating cytokines in patients with CF has been suggested as a contributing factor in the low bone mineral density and low BMI observed in people with CF (207, 208). Chronic airway infection with *Staphylococcus aureus* and *Pseudomonas aeruginosa* give rise to excessive airway and systemic inflammation with resultant lung damage (209, 210). However, measurement of blood cytokines has the advantage that it is less invasive and faster than measurement of BAL cytokines.

The notion that targeting excess inflammation in people with CF may provide clinical benefit is supported by the fact that treatment with the non-steroidal anti-inflammatory agent ibuprofen has been shown to slow the decline in pulmonary function, and result in a reduction in hospitalisation rate and improved ability to maintain weight in people with CF (211, 212). Interestingly, recent data have demonstrated no significant change in sputum cytokines after commencement of ivacaftor, while preliminary work demonstrated a reduction in circulating CRP and immunoglobulin levels in blood after ivacaftor (23). We measured a panel of cytokines using an MSD multiplex ELISA plates to evaluate the impact of a restoration of CFTR function on circulating inflammatory mediators.

Exocrine pancreatic disease

CF has prominent gastro-intestinal and nutritional manifestations including exocrine pancreatic insufficiency, malabsorption of fat- and fat-soluble vitamins, poor weight gain and symptoms of intestinal dysmotility (including constipation, gastro- oesophageal reflux disease and distal intestinal obstruction syndrome). Greater than 85% of patients are exocrine pancreatic insufficient (PI) which is traditionally regarded as irreversible (213, 214). As a result of exocrine pancreatic dysfunction patients with CF suffer from malabsorption of fat- and fat-soluble vitamins and are treated with a traditional high fat CF diet and pancreatic enzyme replacement and vitamin A, D, E, K supplementation. Fat malabsorption can give rise to an inability to maintain weight in patients with CF. In CF low BMI is a recognised risk for increased pulmonary morbidity and mortality with one study demonstrating that patients with CF with a normal BMI had a 21.6% higher mean FEV_1 % predicted than those with a poor BMI (111). Additionally low BMI has been demonstrated to be associated with increased risk of mortality in patients with CF referred for lung transplantation (112).

Faecal elastase 1 (FE-1) is a glycoprotein which is synthesised in the pancreas and excreted in the stool. It is not degraded on passage through the GIT and thus is considered a sensitive marker of exocrine pancreatic function; with levels <100 µg/g stool being considered indicative of severe pancreatic insufficiency, 100-200 µg/ consistent with moderate pancreatic insufficiency and levels > 200µg/g consistent with pancreatic sufficiency (215, 216).

The KIWI study evaluated the safety and efficacy of ivacaftor in very young children aged 2-5 with CF and a gating mutation (25). Interestingly they also measured faecal elastase 1 and blood IRT in this cohort. At baseline 93% of children had a FE-1 less than 50 μg/g consistent with severe pancreatic insufficiency. Notably a 99.8 μg/g significant mean increase in FE-1 was observed after 24 weeks of treatment. Equally of interest was the observation that 26% of children had an FE-1 above the threshold of 200 μg/g considered indicative of pancreatic insufficiency at least once over the course of the study. This raises the biologically plausible possibility that commencement of CFTR modulatory in very young children may augment exocrine pancreatic function. The KIWI study also demonstrated a statistically significant mean absolute improvement in IRT was observed after 24 weeks of treatment; with improvements being observed as early as 2 weeks (25). This chapter aims to evaluate the impact of CFTR modulation on exocrine pancreatic function as reflected by faecal elastase 1.

Endocrine pancreatic disease

Cystic Fibrosis Related Diabetes (CFRD) is a common comorbidity in people with CF with an estimated prevalence of 20% in adolescents and 40-50% in adults with CF (217). CFRD is distinct from Type I and Type II diabetes. Similar to type I DM patients with CFRD have reduced β cell mass in the pancreas and a resultant reduction in insulin production, however, unlike type I DM they do not typically present with diabetic ketoacidosis as they do not suffer from a complete destruction of pancreatic β cellsand as a result have sufficient endogenous insulin production to avoid ketosis (217, 218). An attenuation of insulin secretion after oral glucose is one of the first abnormalities of

glucose metabolism that can be observed in people with CF (218-220).With progression of exocrine pancreatic dysfunction, a reduction in total insulin response is observed. Equally while HbA1c has been shown to correlate with glycaemiccontrol in patients with Type I diabetes in patients with CFRD no correlation was observed between HbA1c and blood glucose level (221). Consequently, it is a less sensitive marker of blood glucose control in patients with CFRD and underestimates how poor glycaemic control has been. Recent works suggests that a HbA1c ≥ 5.8% (40 mmol/mol) in patients with CF is a sensitive but not specific method of screening forthe presence of CFRD and can reduced the need for formal OGTT by 50% (222).

The prevalence of CFRD increases with increasing age with approximately 20% of patient with CF being affected by the age of 20 and 40% of patients by the age of 40 (217). Development of CFRD is associated with more rapid decline in FEV_1 and increased pulmonary exacerbation frequency (223, 224). Lung function has been shown to deteriorate in the years before a diagnosis of CFRD is established in a period when there may be minimal evidence of hyperglycaemia (225, 226). In a 4 year follow up study the rate of lung function decline was demonstrated to be associated with the degree of insulin deficiency with patients with impaired glucose tolerance having an accelerated decline in lung function compare to those with normal glucose tolerance (227). Patients with CFRD and fasting hyperglycaemia had a more rapiddecline in lung function than those with IGT and NGT (227). Early diagnosis andtreatment improves survival in patients with CFRD (217).

A small pilot study of 5 patients with the G551D mutation evaluated the impact of ivacaftor on insulin response and blood glucose levels after an intravenous glucose tolerance test and oral glucose tolerance test (90). A 66-178% improvement in insulin response after oral glucose tolerance testing was observed after one month of ivacaftor (90). Interestingly despite the increase in insulin secretion there was no change in glucose levels during the oral glucose tolerance testing (90). During intravenous glucose tolerance testing four of the patients had a non-statistically significant 51-346% increase in insulin secretion. One patient with new onset anduntreated CF related diabetes did not demonstrate an improvement in acute insulin secretion in response to intravenous glucose (90). Another small study evaluated the insulin secretion and blood glucose levels in two siblings with the S549N gating mutation at baseline and after 16 weeks of ivacaftor treatment (228). The authors demonstrated an improvement from CFRD to indeterminate glycaemia in one sibling and from indeterminate glycaemia to normal glucose tolerance in the second sibling. Equally an increase in early insulin secretion in response to OGTT was also reported, however, the total area under the curve of insulin was not increased (228). However, the clinical implications of these changes in patients remain to be fully established. Hence the final aim of this chapter is to evaluate the impact of ivacaftor on OGTT status, HbA1C and insulin requirements in patients with CFRD.

Methods

Plasma samples were obtained and processed as described in chapter 2. The samples were subsequently thawed on ice. Circulating inflammatory markers were measured in these samples using a multiplex enzyme linked immunosorbent assay (ELISA) (MesoScale Discovery (MSD) platform) in keeping with manufacturer's guidelines (229, 230).

MSD assays provide a rapid sandwich immunoassay for measuring the levels of protein targets within a single, small volume sample. Plates are pre-coated with capture antibodies on independent and well-defined spots, as demonstrated in the figure below (Figure 4.1). The sample and a solution containing detection antibodies conjugated with electrochemiluminescent labels (MSD SULFO-TAG™) are added over the course of several incubation periods. The analytes in the sample bind to capture antibodies which are immobilized on the working electrode surface of the plates.Subsequent recruitment of the detection antibodies by the bound analytes completes the sandwich. MSD buffer is added which creates the appropriate chemical environment for electrochemiluminescence and the plates are loaded into an MSD instrument where a voltage applied to the plate causes the capture labels to emit light. The instrument measures the intensity of the emitted light to provide a quantitative measure of analytes in the sample.

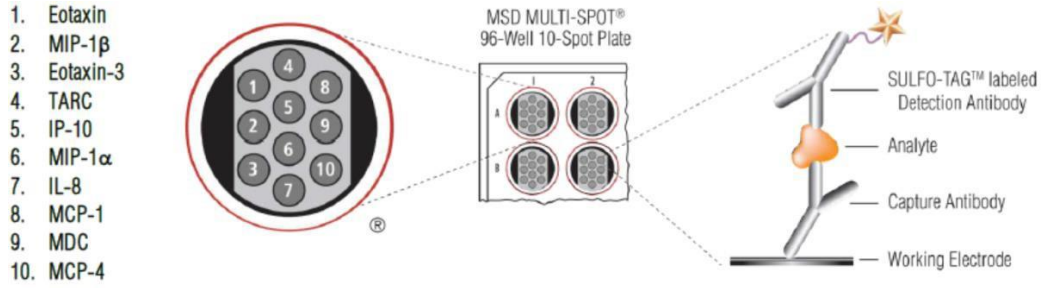


Figure 4.1 Multiplex plate spot diagram showing placement of analyte capture antibodies**.** The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging and in the data files.

Pro-inflammatory panel 1 human kit protocol

Preparation of calibrator dilutions

MSD supplies a multi-analyte lyophilized calibrator that yields the recommended highest calibrator concentration when reconstituted in 1000 µL of Diluent 2. Keep reconstituted calibrator and calibrator solutions on wet ice until use. To prepare 7 calibrator solutions plus a zero calibrator for up to 4 replicates:

1. Prepare the highest calibrator by adding 1000 µL of Diluent 2 to the lyophilized calibrator vial. Mix well by vortexing, then wait a minimum of 5 minutes before using.
2. Prepare the next calibrator by transferring 100 µL of the highest calibrator to 300 µL of Diluent 2. Mix well by vortexing. Repeat 4-fold serial dilutions 5 additional times to generate 7 calibrators.
3. Use diluent 2 as the zero calibrator.

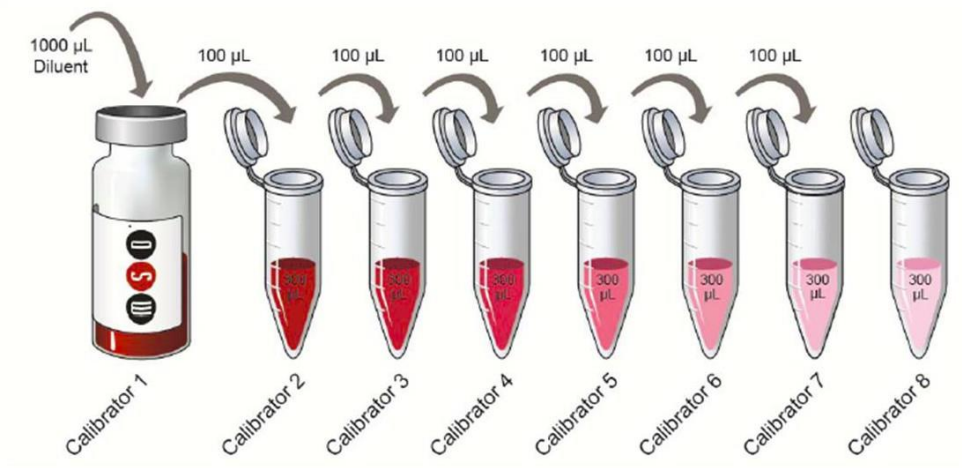


Figure 4.2 Preparation of calibrator dilutions

Dilute samples

Dilute samples with Diluent 2. For human serum, plasma and urine samples, MSD recommends a minimum 2-fold dilution.

Prepare detection antibody solution

MSD provides each detection antibody separately as a 50X stock solution. The working solution is 1X. Prepare the detection antibody solution immediately prior to use. For 1 plate, combine the following detection antibodies, and then add 2400 µL of Diluent 3:

- 60µL of SULFO-TAG Anti-hu IFN-γ Antibody
- 60µL of SULFO-TAG Anti-hu IL-1β Antibody
- 60µL of SULFO-TAG Anti-hu IL-2 Antibody
- 60µL of SULFO-TAG Anti-hu IL-4 Antibody

- 60µL of SULFO-TAG Anti-hu IL-6 Antibody
- 60µL of SULFO-TAG Anti-hu IL-8 Antibody
- 60µL of SULFO-TAG Anti-hu IL-10 Antibody
- 60µL of SULFO-TAG Anti-hu IL-12p70 Antibody
- 60µL of SULFO-TAG Anti-hu IL-13 Antibody
- 60µL of SULFO-TAG Anti-hu TNF-α Antibody

Prepare wash Buffer

MSD provides 100mL of wash buffer as a 20X stock solution in the V-PLEX Plus kit. The working solution is 1X. For one plate combine:

- 15mL of MSD wash Buffer (20X)
- 285mL of deionized water

Prepare Read Buffer

MSD provides Read Buffer T as a 4X stock solution. The working solution is 2X. For 1 plate combine:

- 10mL of read buffer T (4X)
- 10mL of deionized water

Protocol

1. Add 25 µL of assay diluent to each sample/control well and then add 25 µL of neat control or sample. Add 50 µL of each calibrator directly into empty well. Seal the plate with adhesive plate seal and incubate at room temperature with shaking for 2 hours.

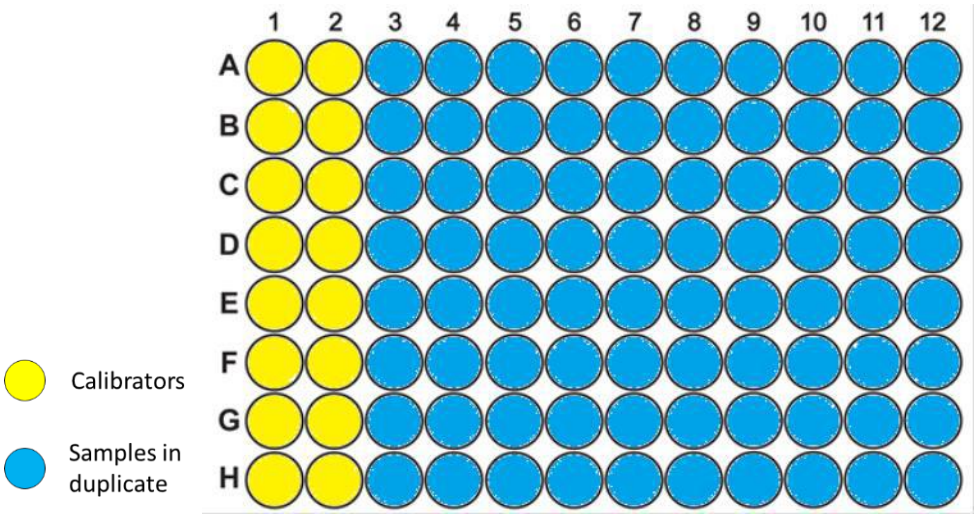


Figure 4.3 MSD plate layout

2. Wash the plate 3 times with at least 150 µL per well of wash buffer. Add 25 µL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature with shaking for 2 hours.
3. Wash the plate 3 times with at least 150 µL per well of wash buffer. Add 150 µ of 2X read buffer T to each well. Read the plate on the MSD instrument. No incubation in read buffer is required before reading the plate.

Faecal Elastase

14 patients with CF with the G551D mutation gave a stool sample at baseline before commencement of ivacaftor and after treatment. The samples were immediately frozen at - 80°C and FE-1 levels were measured using the ScheBO Pancreatic Elastase 1 in Stool test kit (Schebo Biotech, Germany). Similarly, this kit employs a short extraction procedure prior to performing a sandwich ELISA. Clinical data was collected at each visit.

Protocol:

1. Prepare the sample -/ washing buffer and the extraction buffer.
2. Extract and homogenise stool.
3. Dilute stool extract in sample -/ washing buffer.
4. Pipette 50 µl blank, standards, control and samples in duplicate into the ELISA strips.
5. Incubate 30 minutes at room temperature.
6. Wash
7. Add 50 µl anti E1-bio and POD-Streptavidin-Complex (ready to use). Incubate for 15 minutes at room temperature in the dark.
8. Wash
9. Add 100 µl of substrate solution (ready to use). Incubate for 15 minutes at room temperature in the dark.
10. Add 100 µl of stop solution. Read the plate at OD 405 or OD 405-OD492.
11. Evaluate with standard curve using a log-log scale.

Table 4.1 Faecal elastase interpretation	
Faecal elastase level	**Interpretation**
< 100 µg/g	Severe pancreatic insufficiency
100-200 µg/g	Moderate pancreatic insufficiency
>200 µg/g	Pancreatic Sufficient

OGTT and HbA1c

Retrospective annual assessment data from 24 adult patients (age ≥ 16 years) with cystic fibrosis with the G551D mutation who had an oral glucose tolerance test (OGTT), and HbA_1C measured in the year before and the year after commencing ivacaftor was collected and analysed. Fasting glucose and two-hour post prandial glucose levels were recorded. Patients were categorised as having normal glucose tolerance, impaired glucose tolerance (2-hour postprandial glucose 7.8 – 11.0 mmol/l), impaired fasting glucose (fasting glucose 6.1 – 6.9 mmol/l) or Cystic Fibrosis Related Diabetes (CFRD). Six of these 24 patients had pre-existing CFRD and the average insulin requirementsper day and the insulin units per kg of weight were collected in this cohort before and after treatment with ivacaftor.

Clinical data including FEV_1 % predicted, age, gender, and weight were also collected for the year before and the year after commencing treatment. This ivacaftor cohort was matched in a 2:1 manner with a control cohort of 48 age and gender matched patients with cystic fibrosis not receiving ivacaftor. OGTT, fasting lipid profile andHbA1c data for this control cohort were collected for the same matched time period. Four of the patients in the control cohort were excluded from analysis due to

insufficient data. Accordingly, the final control cohort comprised 44 patients. Eight of the patients in the control cohort had pre-existing CFRD.

Statistical Analysis

Data was analysed using SPSS version 22·0 and STATA. Blood cytokine data was $\log_{10}$ transformed before analysis. Paired sample t test was used to evaluate mean change from baseline for normally distributed variables. Paired Wilcoxon signed rank test was used to evaluate median change in non-normally distributed data. Pearson correlation and Spearman Rank correlation coefficients were used to evaluate correlation between clinical parameters, blood biomarkers and Chest CT scores. A chi squared test was used to compare the proportion of patients in different categories before and after ivacaftor.

Results

Significant reductions in circulating $\log_{10}$ IL-6 ($p<0.01$), $\log_{10}$ IL-8 ($p < 0.01$), $\log_{10}$ IL-10 ($p< 0.01$), $\log_{10}$ IL-1β ($p<0.01$) and $\log_{10}$ CRP ($p = 0.015$) were observed after treatment (Figure 4.4). A non-significant reduction in $\log_{10}$ TNF-α was observed after commencement of treatment ($p = 0.06$).

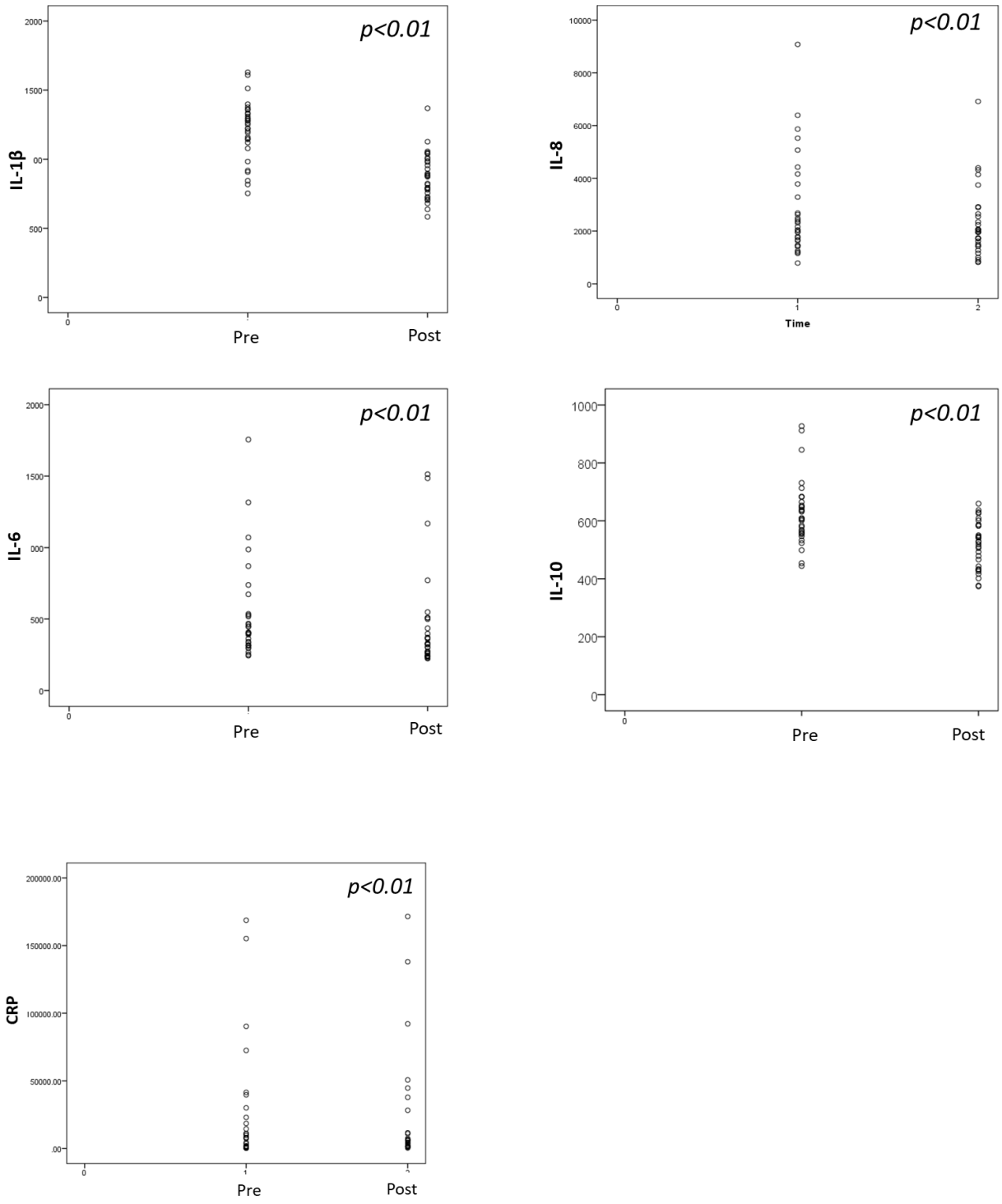


Figure 4.4 Change in blood inflammatory markers after ivacaftor (n=33)

A significant inverse relationship between FEV_1 % predicted and circulating Log_{10} IL-6 was observed at baseline (*r = – 0·61, p < 0·001)* and post therapy (*r = -0·67, p < 0·01*), with patients with a lower FEV_1 having higher concentrations of circulating IL-6 (Figure 4.4). A significant inverse relationship was observed between FEV_1 % predicted and circulating Log_{10} CRP at baseline (r = -0.67, *p < 0.01*) and post treatment (r = -0.56, *p < 0.01*). There was no linear correlation between the magnitude of change in FEV_1 % predicted and the magnitude of change in circulating Log_{10}IL-6 or Log_{10}CRP (Figure 4.5).

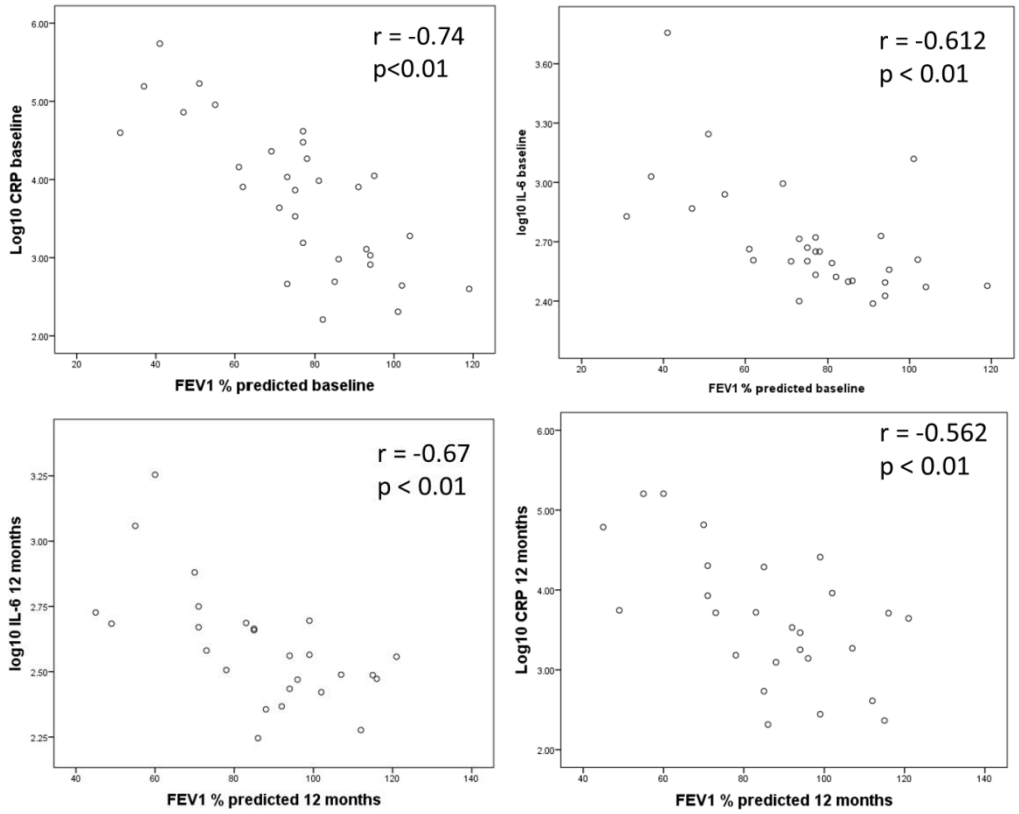


Figure 4.5 Correlation between log_{10} IL-6 and log_{10} CRP and FEV_1 % predicted at baseline and after 12 months of ivacaftor (n=33)

A significant positive correlation was observed between circulating Log_{10} IL-6 and total Bhalla Score at baseline (*r = 0·66, p< 0·01*) and after 12 months (r *= 0·76, p< 0·01*) of ivacaftor; (Figure 4.6). A significant positive correlation was observed between Log_{10}CRP and total Bhalla score at baseline (r = 0·77, *p< 0·01*) and after 12 months of treatment (r = 0·69, *p = 0·004*).

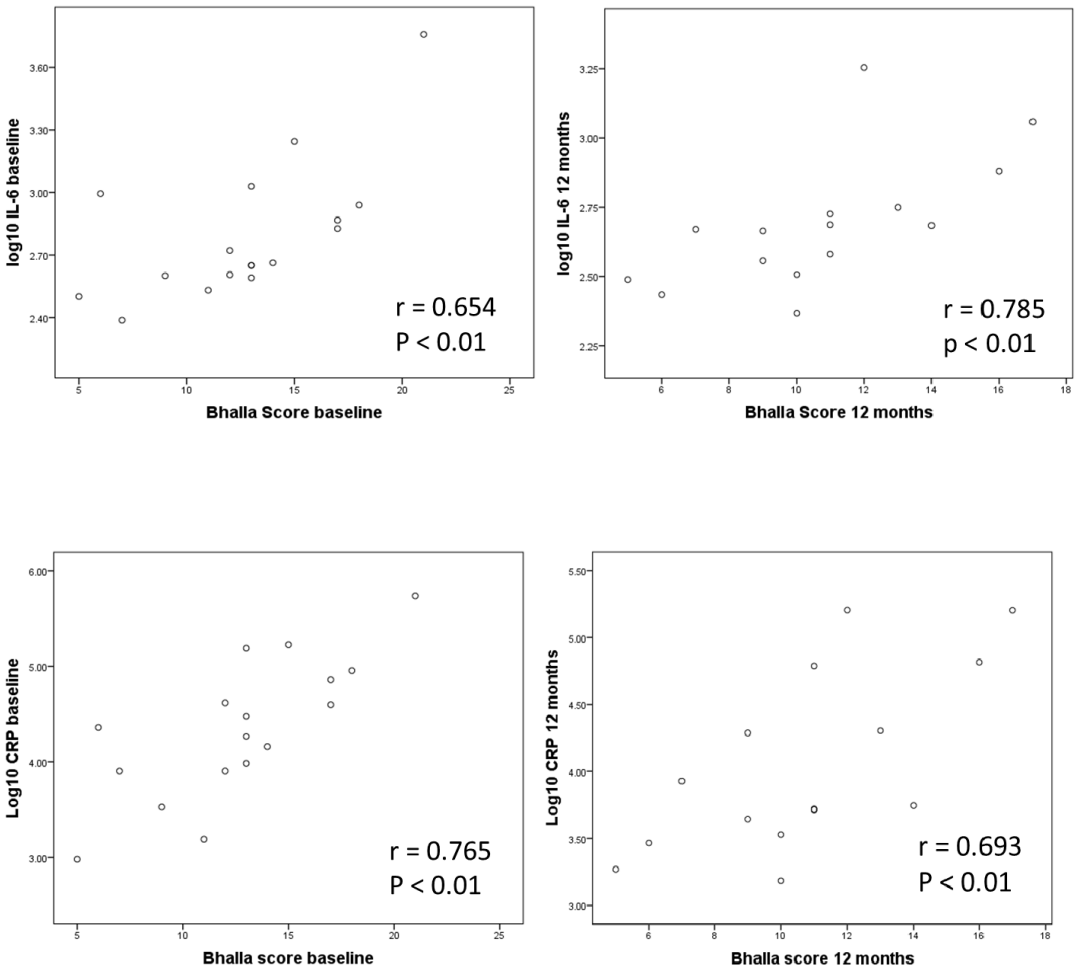


Figure 4.6 Correlation between Log_{10} IL6 and Log_{10} CRP and total Bhalla score at baseline and after 12 months of ivacaftor (n=18)

There was a significant inverse correlation between Log_{10} IL-6 and respiratory symptoms as reflected by CFQ-R respiratory domain at baseline (r = -0.5, *p = 0.036*) andafter 12 months of ivacaftor (r = -0.59, *p = 0.02*) (Figure 4.7). There was a significant correlation between Log_{10} CRP and CFQ-R respiratory domain at baseline (r = -0.608, *p = 0.01*) and after 12 months of ivacaftor (r = - 0.56, *p = 0.03*).

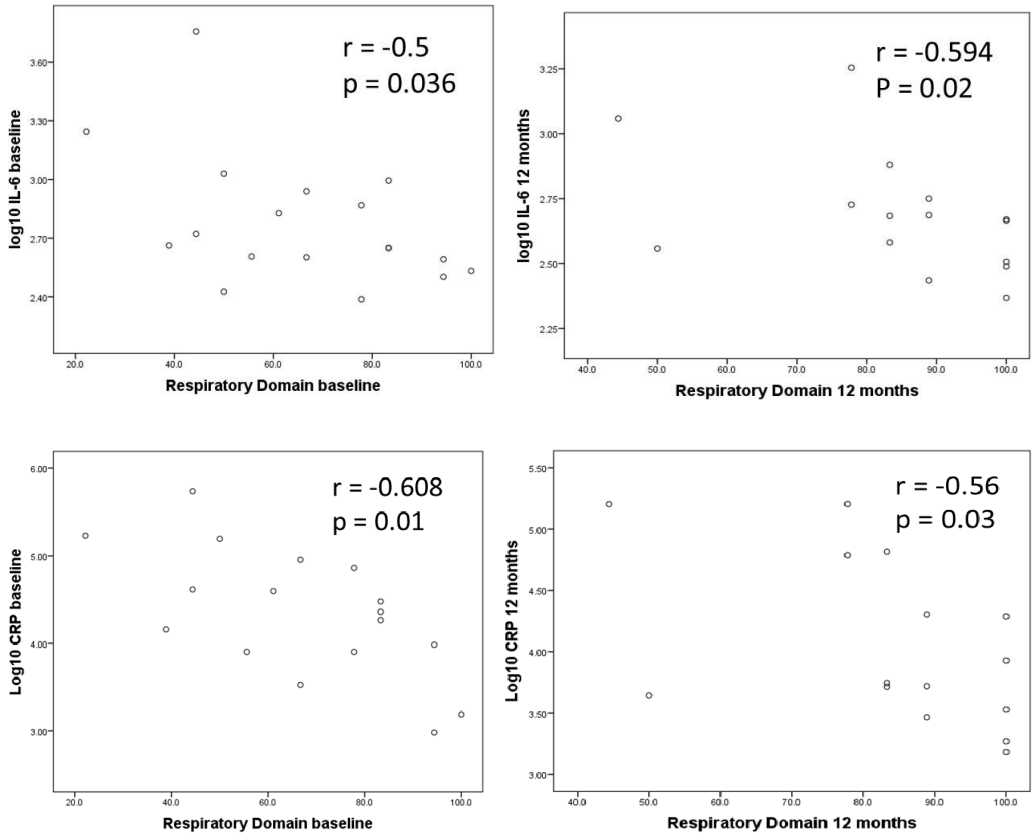


Figure 4.7 Correlation between Log_{10} IL-6 and Log_{10} CRP and CFQ-R Respiratory Domain at baseline and after 12 months of ivacaftor

Circulating IL-6 and IL-8 and blood CRP levels showed a significant inverse correlation with change in community richness (Figure 4.8) (ρ = -0.589 [*p=0.001*]; ρ=-0.417 *[p=0.027*]; ρ=-0.455 [*p=0.018*], respectively). There was also a significant inverse

relationship between IL-6 (ρ=-0.405; *p=0.0330*) and IL-8 (ρ=-0.433; *p=0.021*) and community diversity; however, CRP (ρ=-0.221; p=*0.026*), did not show significant association with levels of community diversity (Figure 4.8). No association was observed between ecological indices (taxonomic richness and community diversity) and TNF-α (ρ=-0.328 [*p=0.088*]; ρ=-0.368 [*p=0.054*]) and IFN-γ (ρ=-0.270 [*p=0.165*]; ρ=-0.150 [*p=0.445*]), respectively.

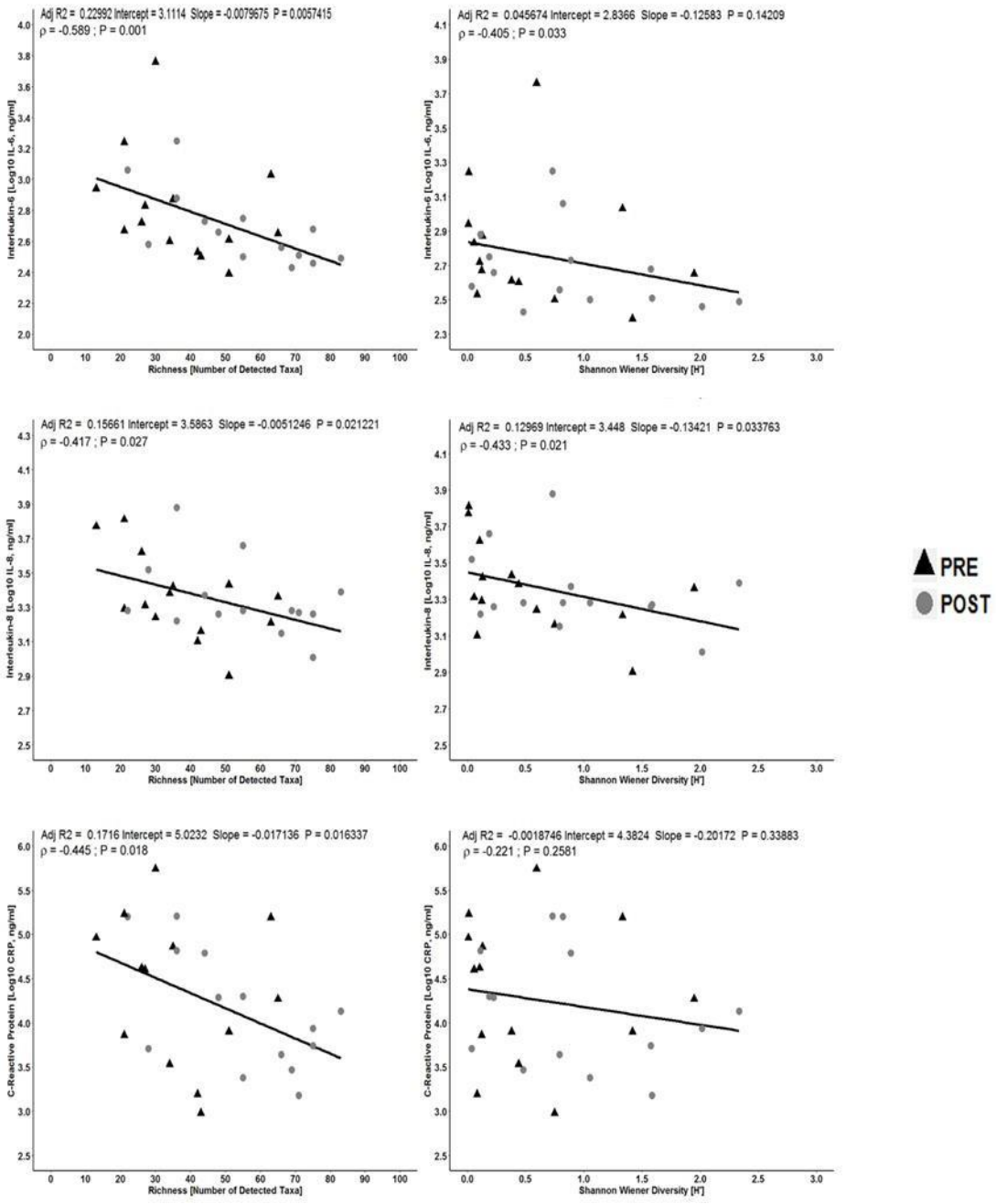


Figure 4.8 Correlation between taxonomic richness and Shannon–Wiener diversity [H'] and markers of inflammation (IL-6 and IL-8) within the whole meta-community (n=14; filled triangle denotes Pre-treatment samples; filled circle denotes POST-treatment samples). Line indicates linear relationship between variables and the Spearman's correlation coefficient (ρ) is shown (*P<0.05* denotes statistical significance).

Pseudomonas species positivity correlated with increased levels of blood IL-8 levels. An inverse correlation was observed between IL-6 and IL-8 and *Streptococcus* positivity species.

Table 4.2 correlation between blood cytokines and main detected genera (n=14)						
	IL-6		IL-8		CRP	
Genera	r	*p*	r	*p*	r	*p*
Pseudomonas spp.	0.29	0.12	0.38	0.04	0.3	0.12
Haemophilus spp.	0.04	0.86	0.09	0.71	0.09	0.68
Streptococcus spp.	-0.37	0.04	-0.48	0.01	-0.34	0.07
Rothia spp.	-0.49	<0.001	-0.43	0.02	-0.44	0.01
Prevotella spp.	-0.40	0.03	-0.24	0.19	-0.45	0.02
Veillonella spp.	-0.37	0.04	-0.06	0.77	-0.38	0.04

FE-1

There was no statistically significant or clinically significant change in faecal elastase 1 after commencement of ivacaftor (*p = 0.614*) (Figure 4.9).

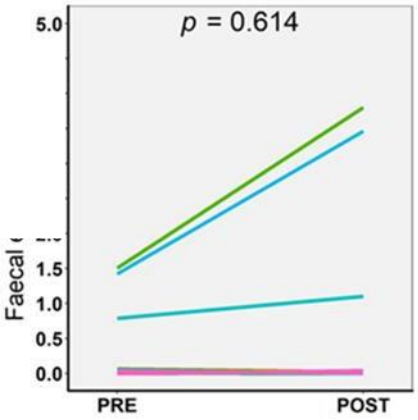


Figure 4.9 Faecal elastase level before and after ivacaftor (n=14)

All patients had a faecal elastase 1 level less than 100 µl/l before and after ivacaftor: consistent with severe pancreatic insufficiency ($p = 1.0$).

CFRD

Table 4.3 summarises baseline characteristics of the ivacaftor and control groups. The mean age of the ivacaftor cohort was 27.21 years and 27.42 years for the control cohort. 58% of those in the ivacaftor group were male and 53% in the control group.

Table 4.3 Mean (SD) baseline characteristics of the Ivacaftor and Control cohorts

Baseline characteristics	Ivacaftor group N = 24	Non-Ivacaftor group N = 44
Male	58%	53%
Age	27.21 (5.8)	27.42 (5.4)
FEV1 % predicted	60.13 (21.7)	68.75 (22.9)
Weight	60.45 (12.9)	64.88 (10.5)
BMI	20.97 (2.94)	21.85 (4.77)
Cholesterol	3.2 (0.88)	3.8 (0.91)
HDL	1.12 (0.28)	1.39 (0.45)
LDL	1.73 (0.53)	2.0 (0.68)
Triglycerides	0.9 (0.48)	1.11 (0.48)
Fasting glucose	5.4 (1.34)	4.99 (0.6)
2 hour postprandial glucose	4.4 (2.9)	5.56 (3.08)
HbA1c	46.92 (19.8)	44.36 (9.3)

In the 12 months after commencement of ivacaftor a 10.96% significant mean absolute increase in FEV_1 % predicted ($p < 0.01$) and 3.98 kg mean absolute increase in weight

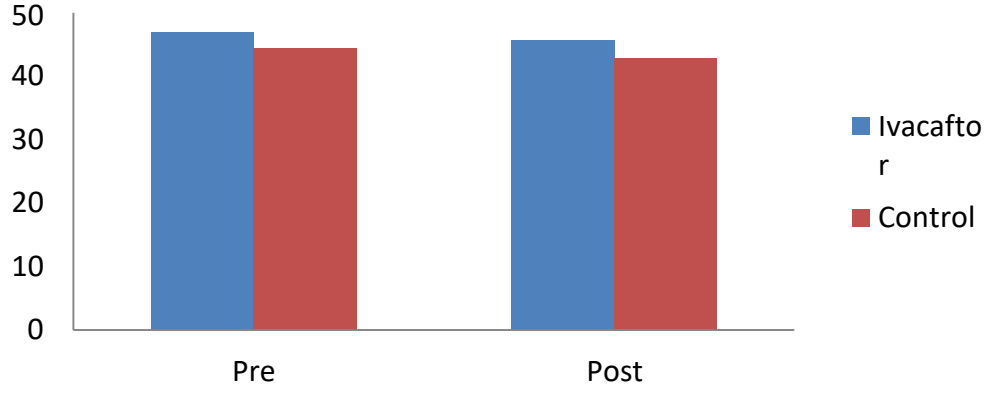
(*p<0.01*) were observed. A 4.5% mean absolute reduction in FEV_1 % predicted (*p<0.01*) and 0.079 kg mean absolute increase in weight (*p = 0.88*) were observed in the control cohort over the same time period. A significant 16.47% relative mean increase in FEV_1 was observed in the ivacaftor group compared to the control cohort (t = 7.8, *p <0.01*). A significant 3.9 kg relative mean increase in weight was observed in the ivacaftor group compared to the control group (t = 4.22, *p <0.01*).

There was a significant reduction in the percentage of patients underweight and an increase in the percentage of patients who were classified as overweight after ivacaftor (*p = 0.034*).

Glucose tolerance

There was no significant change in HbA1c within the ivacaftor group (mean change -1.17, *p = 0.46*) or compared to the control group (mean difference = 0.55, t = -0.39, *p = 0.7*) in the year after commencement of ivacaftor (Figure 4.10).

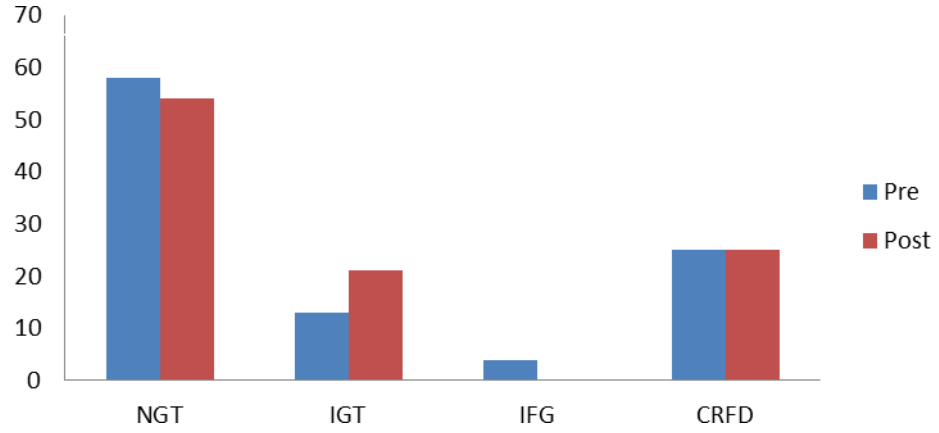
Figure 4.10 Mean HbA1c before and after ivacaftor



Oral Glucose Tolerance Test

There was no significant change in fasting glucose (mean change -0.03, *p = 0.77*), or two-hour post-prandial glucose in the year after commencement of treatment (mean change 0.24, *p = 0.74*). There was no significant difference in the change in fasting glucose (mean difference -0.14, t = -0.6, *p = 0.55*) or 2 hour post prandial glucose in the ivacaftor group compared to the control group (mean difference -0.14, t = -0.19, *p = 0.85*) in the year after ivacaftor. There was no significant difference in the change in There was no significant change observed in OGTT status post treatment within the ivacaftor (Figure 4.11).

Figure 4.11 Oral glucose tolerance test category before and after ivacaftor (n=24)

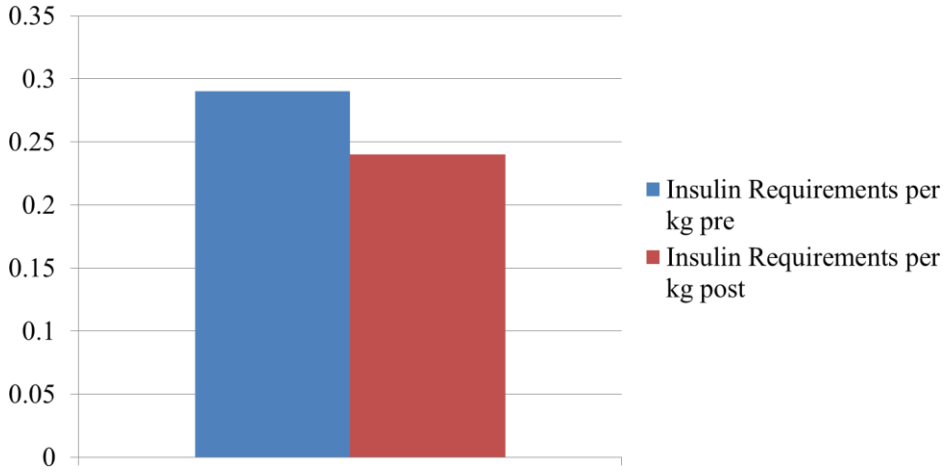


Insulin requirements

In the 6 patients with CFRD no significant change in either total insulin requirements (*p = 0.42*) or units of insulin per kilogram of weight (*p = 0.25*) were observed after

treatment (Figure 4.12). There was no significant difference between change in total insulin requirements per day in the ivacaftor group compared to the control group (*p = 1*). There was no significant difference between the change in insulin requirements per kg in the ivacaftor group compared to the control group (*p = 0.052*).

Figure 4.12 Median insulin requirements per kilogram weight before and after ivacaftor (n=6)



Discussion

We observed a significant reduction in several circulating inflammatory markers. This "real-world" finding is consistent with the results from a group of patients in the clinical trials that demonstrated a significant reduction in the circulating neutrophil count as well as IL-8, CRP, and IgG levels after 24 weeks of treatment (231). That said, another real-world cohort failed to demonstrate a significant change in sputum cytokine levels in patients with CF treated with ivacaftor (23). Inflammation in the CF lung is characterized by an excess influx of polymorphonuclear neutrophils (232, 233).

Exaggerated inflammation develops early in the course of CF, with elevated levels of inflammatory markers

being demonstrated in the lungs of infants with CF in the absence of overt infection (232, 233). Previous studies have demonstrated elevated levels of circulating inflammatory markers, including IL-2, IL 6, IL-8, and TNF-a, in patients with CF compared with control cohorts (204, 234, 235). Similarly, some studies have demonstrated a reduction in circulating inflammatory markers after antibiotic therapy for pulmonary exacerbations (236). Other medications have also demonstrated reduction in inflammatory markers in CF. A 4-year study in a paediatric cohort will with mild-moderate lung disease demonstrated that alternate day prednisolone was associated with improved FEV_1, weight gain and a lower hospitalisation rate compared to placebo (237-239). A further paediatric study demonstrated a superior FEV_1 in patients treated with 1mg/kg of prednisolone on an alternate day basis. Benefits were greater in those who were infected with *Pseudomonas aeruginosa*. Azithromycin use resulted in increased FEV_1, a reduction in pulmonary exacerbations, and improved symptoms and weight gain in patients with CF who were chronically colonised with *Pseudomonas aeruginosa* in one study (120). Further work in patients with CF demonstrated that azithromycin resulted in a reduction in circulating neutrophil count and markers of systemic inflammation including CRP and calprotectin in people with CF (240). Treatment with dornase alpha has been shown to be associated with lower BAL neutrophil counts, IL-8 and elastase levels compared to controls (241).

Whether the reduction seen in our study is solely due to a decrease in pulmonary exacerbations observed in our cohort or augmented by potential changes in immune function post restoration of CFTR function remains to be evaluated given that studies have demonstrated improved neutrophil function after ivacaftor therapy (242, 243).

There was no significant change in faecal elastase 1 after ivacaftor. This may reflect testing in an older adult cohort with established irreversible pancreatic insufficiency.

Previous studies have suggested an improvement in insulin secretion, with some cases reporting a resolution of CFRD after commencement of CFTR modulatory therapy (88). In a retrospective single centre cohort, we observed no significant change in HbA1c, fasting glucose, or 2-hour post prandial glucose in a cohort of patients treated with ivacaftor. However, there is some controversy around HbA1c utility in this setting given that it may fail to identify patients with impaired glucose tolerance that may benefit from a period of close monitoring (222, 244). Equally HbA1c appears to have a higher false negative rate in patients with more severe disease who would benefit from early initiation of insulin therapy (245). The role of HbA_1c in the screening for CFRD remains to be fully established. Similarly, when compared to a cohort of patients not receiving CFTR modulatory therapy there was no significant change in the above parameters in the first year after treatment. In a cohort of 6 patients with pre-existing CFRD treated with ivacaftor and a cohort of 8 patients with CFRD not on ivacaftor no significant within-group or between group change in total insulin requirements or insulin requirements per kg weight were observed after commencement of ivacaftor. It remains to be established if early long-term treatment with ivacaftor will delay the

onset on CFRD, however, in the short term in an adult cohort our data suggests that CFTR modulatory therapy does not change HbA1c, glucose tolerance or insulin requirements.

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Limitations

A limitation of the faecal elastase analysis is the small sample size. However, given that the global prevalence of the G551D mutation is 4-5% this represents a relatively large sample size. An additional limitation is that it is predominantly an adult, cohort. It is possible that treatment in younger children with a shorter duration of pancreatic insufficiency may result in some restoration of exocrine pancreatic function after ivacaftor. A limitation of the oral glucose tolerance analysis is that it is retrospective data with the associated biases. An additional limitation is that insulin levels were not measured and while no significant change in oral glucose tolerance or HbA1c were observed it is possible that there were changes in insulin secretion.

Conclusion

There was no significant change in exocrine pancreatic function in an older cohort of patients with CF after commencement of ivacaftor suggesting that it does not restore pancreatic function. This may reflect established long-standing pancreatic destruction which is irreversible. There was no significant change in oral glucose tolerance status or insulin requirements thus the clinical impact of ivacaftor on glucose metabolism remains to be fully evaluated.

Chapter 5

Back to the future: CFTR modulation and the CF gut

Introduction

While respiratory compromise is the leading cause of death in PWCF, when first described by Dorothy Hansine Andersen in 1938 as cystic fibrosis of the pancreas, gastrointestinal manifestations predominated, and PWCF died of malnutrition and gastrointestinal complications in the first few months of life (151). Patients with CF may present at birth with meconium ileum (MI). This consists of bowel obstruction as a result of the accumulation of inspisated mucus and faecal material and abnormal GI motility. Prevalence is approximately 10 - 15% (246, 247). Older patients with CF may alsosuffer from intermittent episodes of complete or partial bowel obstruction due to accumulation of faeces, especially at the ileocaecal junction, referred to as distal intestinal obstruction syndrome (DIOS). Prevalence is estimated at 10-47% (248). ESPGHAN further define this based on severity as complete DIOS (C DIOS) or Incomplete/Impending DIOS (I DIOS) (246, 247). C DIOS is the combination of evidence of complete intestinal obstruction (bilious vomiting and/or abdominal radiography demonstrating fluid levels in the small intestine), faecal mass in the ileo-caecum and abdominal discomfort and/or distension. I DIOS is defined as a short history of abdominal discomfort and/or distension, and a faecal mass in the ileo-caecum in the absence of features of complete obstruction (246, 247).

Gastro-oesophageal reflux disease (GORD) is frequently observed in patients with CF, with a prevalence of up to 80% (249). A variety of factors have been postulated to contribute to GORD in patients with CF including reduced gastric motility and reduced oesophageal sphincter relaxation (250, 251). Importantly there is an increased risk of

gastric content micro aspiration in CF. The presence of GORD in patients with CF is associated with an increased cough frequency and increased decline in lung function (250, 251). An association has been demonstrated between the presence of bile acid in sputum and increased airway inflammation and airway *Pseudomonas aeruginosa* colonisation (251).

Gut inflammation

Inflammation of the gastro-intestinal tract is prominent in CF. Pilcam studies have demonstrated increased inflammation in the CF gut compared to controls (252). Analysis of gut lavage samples from children with CF demonstrated increased levels of inflammatory markers including IgG, IgM, neutrophil elastase, Il-1β and IL-8. An exaggerated influx of mononuclear cells into the lamina propria of duodenal mucosal specimens in children with CF has been observed with a consequent elevated expression of IFN-γ, IL-2, ICAM-1 and transferrin receptors has been described (253). Calprotectin is present in neutrophils, monocytes and macrophages. Faecal calprotectin is present in high levels in individuals with intestinal inflammation including Crohn's disease and has been shown to be elevated in PWCF compared to controls (252, 254, 255). Previous work in patients with inflammatory bowel diseasehas demonstrated a correlation between faecal calprotectin levels and the grade of mucosal inflammation on histological evaluation (254). That work also demonstrated that faecal calprotectin was a better predictor of active mucosal inflammation than

other markers (254). Treatment with a probiotic has been shown to reduce faecal calprotectin levels in patients with CF (256).

Faecal lactoferrin is an innate immunity protein, which is produced by neutrophils and considered to be both a sensitive and specific a marker of gastrointestinal tract inflammation (257). Faecal lactoferrin is rapidly released into the gastrointestinal tract during periods of gut inflammation. Work in patients with Crohn's disease has demonstrated a reduction in faecal lactoferrin levels after successful treatment with anti-TNFα therapy (258, 259). Given the excess inflammation previously reported in the CF gut we evaluated the impact of ivacaftor on faecal calprotectin and lactoferrin before and after treatment.

Gut microbiota

The human gut is populated by a diverse population of microbes; containing thousands of organisms which confer potential health benefits. A variety of factors can alter this microbiome, including antibiotic treatment and infection with various pathogens. There is increasing evidence to support the hypothesis that the gut microbiota plays a role in host metabolic and immunological function (260, 261). Alterations in the gut microbiota have been postulated to play a role in many disorders of the gastrointestinal tract; including irritable bowel syndrome and inflammatory bowel disease (262). Interestingly GI dysbiosis has been implicated in many diseases outsideof the gastrointestinal tract, including liver disease and conditions which involve inflammation and metabolic disorders including cardiovascular disease (263-265).

Work in infants has demonstrated that GI dysbiosis in early life may give rise to persisting abnormalities of systemic immunity, metabolism and an increased vulnerability to respiratory tract infections (264, 266).

The gut microbiota of patients with CF differs from that of healthy controls. It has been suggested that the gut microbiota may play a role in the occurrence of intestinal and general inflammation observed in patients with CF (261, 267). These alternations in the gut microbiota appear to be present from early life in children with CF. Previous work in a paediatric cohort demonstrated significant differences in the gut microbiota of children with CF compared to a control group with reduced levels of *Bacteroides uniformis, Bacteroides vulgatus, Bifidobacterium adolescentis, Bifidobacterium catenulatum and Faecalibacterium prasunitzii (268)*. The gut microbiota was further perturbed in children with CF who were on antibiotic treatment. Treatment with a probiotic resulted in a partial restoration of the intestinal microbiota. Interestingly a correlation was observed between a lower gut microbial richness and intestinal inflammation (268). Additionally, patients with CF have a higher frequency of carriage of C. difficile, which may arise as a result of recurrent antimicrobial treatment (269).

There is evidence to suggest that the gut microbiota may impact on the CF lung given than administration of probiotics to patients with CF has been associated with an improvement in FEV_1, a decrease in pulmonary exacerbations, and a decrease in hospital administrations (270, 271). A variety of factors may contribute to an altered gut microbiota in people with CF including CFTR dysfunction, recurrent courses of oral, intravenous and inhaled antibiotics, pancreatic enzyme replacement, gut inflammation

and a high fat diet. Therefore, we evaluated the impact of CFTR modulation on the gut microbiota.

Methods

Subjects

Fourteen adult Ivacaftor-naive patients with Cystic Fibrosis consented to participate. They were assessed at baseline before commencing ivacaftor and on a three-monthly basis in routine CF clinic in line with local requirements for monitoring during the first year of treatment with Ivacaftor.

Patient Assessment

Participants attended the Cystic Fibrosis Day Centre when clinically stable and free from pulmonary exacerbation before commencing ivacaftor, as described in earlier chapters. Patients completed the Cystic Fibrosis Questionnaire Revised (CFQ-R is a CF specific quality of life questionnaire consisting of domains scored from 0 -100 with higher scores indicating that the patient is feeling better) (122) were recorded at baseline. Numbers of antibiotic courses (Intravenous (IV)) for pulmonary exacerbations were documented prospectively for 3 - 12 months after commencement of Ivacaftor and for the same matched period in the previous year depending on the period of patient follow-up. The patients consented to donate a stool sample before commencing Ivacaftor and at routine three monthly follow-up, when possible. Stool samples were collected in sterile universal containers and frozen at -80^{o}C for later DNA

extraction, and analysis of faecal calprotectin and faecal lactoferrin measurement. Ethical approval was obtained from the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Intestinal inflammation

Faecal calprotectin and faecal lactoferrin were evaluated in 14 baseline samples each with at least one follow-up time-point after ivacaftor treatment.

Calprotectin

Principle of the faecal calprotectin ELISA assay:

The test allows for the selective measurement of the Calprotectin antigen by sandwich ELISA. A monoclonal capture antibody (mAb) highly specific to the calprotectin heterodimeric and polymeric complexes respectively is coated onto the microtiterplate. Calibrators, controls and patients' extracts are incubated at room temperature for 30 minutes. After a washing step a detection antibody (Ab) conjugated to horseradish peroxidase (HRP) detects the calprotectin molecules bound to the monoclonal antibody coated onto the plate. After incubation and a further washing step, tetramethylbenzidine (TMB) will be added (blue colour formation) followed by a stopping reaction (change to yellow colour). The absorption is measured at 450nm.

Faecal calprotectin methods:

Standard stool extraction procedure

1. Label and weigh the empty polypropylene tube together with the inoculation loop.
2. Take out 50 to 100mg of the stool sample by means of the inoculation loop and place it into the pre-weighted tube.
3. Calculate the net amount of sample, break off the inoculation loop and leave the lower part of the loop in the tube.
4. Add extraction buffer according to the formula

   X mg stool x 49 = y µl extraction buffer (e.g. 50mg stool + 2450 µl buffer) to the tube and close the tube
5. Homogenise the sample on a multi tube vortexer by vigorous shaking at highest speed for 30 minutes.
6. Transfer the homogenate into a 2mL Eppendorf tube and centrifuge in a micro centrifuge for 5 minutes at 3000g.
7. Take the supernatant into a fresh, labelled tube and continue with the ELISA procedure.

Assay procedure:

1. Allow the reagents to equilibrate to 18-28°C prior to use. Only dilute stool extracts. Standards and controls are ready to use.
2. Dilute the stool extracts 1:50 with incubation buffer (e.g. 20µL extract and 980µL incubation buffer) and mix well. Let the samples equilibrate for at least 5 minutes at 18-28° prior to proceeding to the next step.

3. Prepare a plate with sufficient strips to test the required number of calibrators, controls and dilutes samples. Remove excess strips from the holder and re-seal them inthe foil pouch together with the desiccant packs without delay. Store refrigerated.
4. Wash the coated wells twice using at least 300µL of wash buffer per well. Empty the wells and tap the plate firmly onto blotting paper. For every of the three wash steps a minimal incubation time of at least 20 second of the wash buffer in the wells must be ensured.
5. Pipette 100µL of incubation buffer (blank) and pipette 100µL of calibrator A-E into the respective wells. Pipette 100µL of each diluted sample into the subsequent wells.
6. Cover the plate with a plate sealer, and incubate for 20 + max 5 minutes on a plate rotator set at 450 rpm at 18-28°C.
7. Remove and discard the plate sealer. Empty the wells and wash three times using at least 300µL of wash buffer per well. Empty the wells and tap the plate firmly onto blotting paper.
8. Pipette 100 µL of enzyme label to each well.
9. Cover the plate with a plate sealer and incubate for 30 +/- 5 minutes on a plate rotator set at 450rpm at 18-28°C.
10. Remove and discard the plate sealer. Empty the wells and wash five times using at least 300µL of wash buffer per well. Empty the wells and tap the plate firmly onto blotting paper.
11. Allow the TMB substrate solution to equilibrate to 18-28°C. Pipette 100µL of the TMB substrate solution to all wells.
12. Cover the plate with a plate sealer, protect the plate from direct light and incubate for 15 +/- 2 minutes on a plate rotator set at 450 rpm at 18-28°C.

13. Pipette 100µL of stop solution to all wells. Remove air bubbles with pipette tip. Proceed to step 14 within 30 minutes.
14. Read the absorbance at 450 nm in a microtiter plate reader.

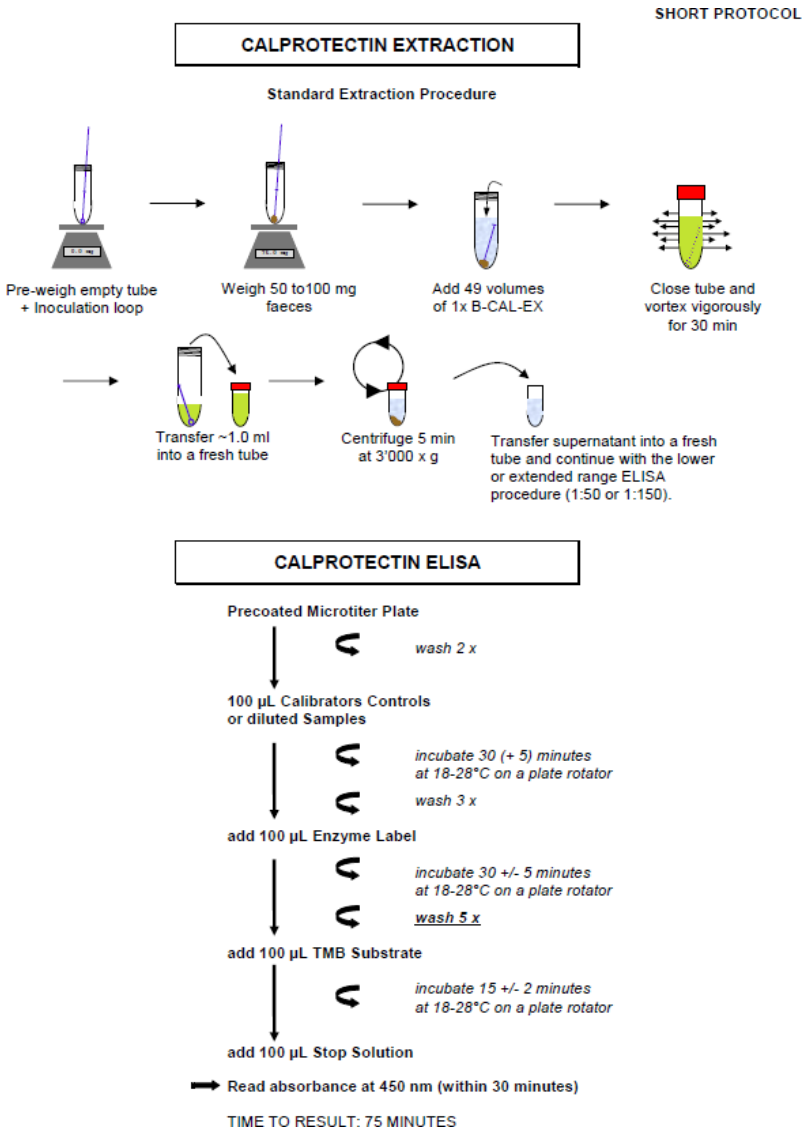


Figure 5.1 Faecal calprotectin extraction procedure

Results and calculation

The absorbance is read at 450 nm in a microtiter reader for each calibrator, control and sample using a 4 PL fit with blank subtraction and have the concentration of the samples calculated.

Table 5.1 Faecal calprotectin interpretation	
Calprotectin level	**Interpretation**
< 50 µg/g	Normal
50 – 200 µg/g	Elevated – may represent mild organic disease such as inflammation caused by, mild diverticulitis and IBD in remission
>200 µg/g	Suggestive of active organic disease with inflammation in the GIT.

Lactoferrin

The IBD-SCAN test uses antibodies to human lactoferrin. The micro-assay wells supplied with the kit contain immobilised polyclonal antibody against lactoferrin. The detecting antibody consists of polyclonal antibody conjugated to horseradish peroxidase. In the assay, standards and serial dilutions of faecal specimens are transferred to the micro-assay wells. If detectable levels of lactoferrin are present in the specimen, the lactoferrin will bind to the immobilised antibody. After incubation, the wells are washed, and the antibody conjugate is added. The conjugate will bind to the lactoferrin bound during the first incubation phase. Any unbound material is removed during a second series of wash steps. Following the addition of substrate, a

colour is detected due to the enzyme-antibody-antigen complexes that form in the presence of lactoferrin. The absorbance measured is directly proportional to the concentration of lactoferrin present. Lactoferrin standards ranging from 6.25 to 100ng/mL are used to generate a standard curve. By plotting absorbance values versus lactoferrin concentrations the lactoferrin concentration in a test sample can be determined.

Preliminary preparations:

1. Remove all reagents from the kit box to warm to room temperature before use.
2. Prepare 1X wash solution. The wash solution is supplies as a 20X concentration (a precipitate may be noticed). It should be mixed and diluted to a total volume of 1 L by adding 50mL of the concentrate to 950mL of deionised water. Label the bottle. Store any unused 1X wash solution between 2-8°C.
3. Prepare 1X diluent. The diluent is supplied as a 10X concentrate (a precipitate may be noticed). It should be mixed and diluted to a total volume of 400mL by adding 40mL of the concentrate to 360mL of deionised water. Label the bottle. Store any unused 1X diluent between 2-8°C.
4. Micro assay plate preparation. Each strip contains 8 wells coated with polyclonal antibody specific for lactoferrin. Each specimen or control will require one of these coated wells. Avoid contact with the bottom of the wells because this is the optical window for ELISA readers. Micro-assay wells not used must be returned to the foil bag and carefully resealed with desiccant.

Collection of specimens and preparation of dilutions:

Collect faecal specimen into a clean airtight container with no preservatives. Mix (vortex) specimens thoroughly prior to performing the assay. This includes complete mixing of the specimen prior to transfer to diluent as well as complete mixing of the diluted specimen prior to performing the assay

Prepare diluted specimen:

1. Set up three plastic tubes for each test specimen. Label the tubes 1-3. For each specimen, add 450µL of 1X diluent to each of the three tubes using a pipette. Using a transfer pipette, measure 50µL of faeces (wipe excess specimen from the pipette tip) and add to tube number 1. Discard the transfer pipette following the initial specimen dilution. For liquid/ soft faecal specimens – using a transfer pipette, carefully add 50 µL (first mark or top of flared tip) of the specimen to tube number 1 (1:10 dilution of specimen) and mix well using a vortex mixer. Measure specimen carefully to ensurethe correct dilution. For formed/solid faecal specimens weigh 0.05g or fill the flared tip of the transfer pipette (50µL) and add to tube number 1 (1:10 dilution of specimen) and mix well using a vortex mixer. Measure specimen carefully to ensure correctdilution.
2. Transfer 50µL from tube 1 into tube 2 using a new transfer pipette and vortex (1:100 dilution of specimen).
3. Transfer 50µL from tube 2 into tube 3 using the same transfer pipette (1:1000 dilution of specimen).

Prepare positive control

4. Set up two plastic tubes for the positive control. Add 450µL of 1X diluent to tube number 1 and 950 µL of tube number 2 using a calibrated pipette.
5. Transfer 50µL of positive control to tube number 1 (1:10 dilution of positive control) and mix well using a vortex mixer. Transfer 50µL from tube number 1 to tube number 2using a calibrated pipette (1:200 dilution of positive control).
6. Vortex all tubes for 10 seconds and store between 2°C and 8° until the ELISA is performed. Vortex again before transferring diluted specimens and positive control to the micro-assay wells. This ensures thorough mixing of the specimen and controls.

Test procedure

1. Designate and use 2 wells for each standard, 1 well for the negative control (1Xdiluent), 1 well for the 1:200 dilution of positive control and 1 well for specimen dilutions 1:100 and 1:1000.
2. Using a calibrated pipette, add 100µL of each standard LS1-LS5 to duplicate wells and 100µL of the 1X diluent and positive control to designated wells.
3. Add 100µL from each specimen dilution (1:100 and 1:1000) to separate wells.
4. Cut the adhesive plastic sheet to the size necessary to cover the wells. Cover the wells and incubate them at 37°C +/- 2°C for 30 minutes stationary.
5. Shake out the contents of the assay wells into a discard pan.
6. Wash each well 5 times using the 1X wash solution in a squirt bottle with a fine tipped nozzle, directing the wash solution to the bottom of the well with force (i.e. fill the wells, then shake the wash solution out of the wells into a discard pan). Slap the

inverted plate on a dry paper towel and repeat four times using a dry paper towel each time. If any particulate matter is seen in the wells, continue washing until all the matter is removed.

7. Add 1 drop of conjugate (red cap) to each well. Incubate the wells at 37°C +/- 2°C for 30 minutes stationary.
8. Repeat step number 6. Dispose of paper towels and specimen containers properly.
9. Add 2 drops of substrate (blue cap) to each well. Gently tap the wells to mix the contents. Incubate the wells at room temperature for 15 minutes. Gently tap the wells 1 or 2 times during this incubation period.
10. Add 1 drop of stop solution (yellow cap) to each well. Gently tap the wells to mix and wait 2 minutes before reading. The addition of the stop solution converts the blue colour to a yellow colour which may be quantitated by measuring the optical density at450 nm or 450/620 nm on a microplate ELISA reader. Wipe the underside of each well with a soft paper towel before measuring the optical density. Read within two to ten minutes after adding stop solution.
11. Record absorbance values for the positive control dilution, for the negative control for each specimen dilution and for the standards.
12. Average the acceptable readings of duplicate wells before interpreting results.

Calculation of results

1. An appropriate data reduction computer program, using linear trend/ regression analysis, should be used for optimal estimation of sample values. If a computer program is not available, the data may be plotted using graph paper.

2. Choose the most diluted specimen that gives and OD_{450} or $OD_{450/620}$ value within the standard curve and OD ≥0.1 or 0.06 respectively. If both sample dilutions have absorbance readings greater than the highest concentration of standard, repeat using additional 1:10 dilutions. Conversely, any sample having an absorbance reading less than the lowest concentration of standard should be retested using the 1:10 dilution and if found negative recorded as <1µg/g wet weight.
3. Plot the average absorbance values of the standards on the y axis versus the concentration on the x axis.
4. Perform the linear trend/regression type analysis and determine if the R^2 value is > 0.98.
5. Instruct the program to produce the equation for the plotted line. The equation should fit the equation of a line which is Y = MX + B, where Y = OD_{450} or $OD_{450/620}$ of the sample, M = slope, B = Y intercept and X = concentration of the unknown sample.
6. Solve the equation for X to determine the concentration of lactoferrin in the specimen.
7. Multiply the value of the unknown sample by the dilution factor.
8. Divide by 1000 to convert to ng/mL to µg/mL (approximately µg/g wet weight).

Table 5.2 Faecal lactoferrin interpretation	
Lactoferrin value	Interpretation
0 – 7.24 µg/mL (g) faeces	Baseline (normal)
≥ 7.25 µg/mL (g) faeces	Elevated

Gut microbiota

Stool samples were stored at -80°C for up to 9 months and then thawed on ice, prior to DNA extraction. DNA was purified from thawed faecal samples using the Repeated Bead Beating (RBB) DNA extraction method previously described by Yu and Morrison (2004). Removal of RNA, protein and purification of the DNA was completed using components of the QIAGEN QIAamp DNA Stool Mini kit. DNA was subsequently stored at -20°C. The microbiota composition of the samples was established through amplicon sequencing of the commonly used 16S r RNA V3/V4 region according to the 16S metagenomics library prep guide (Illumi-na). Each genomic DNA template was PCRed twice to complete complex library preparation; 16S rRNA gene specific primers with integrated Illumina adaptor overhang nucleotide sequences (Forward Primer 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were used to initially isolate the 16S r RNA V3/V4 region, Illumina Nextera XT (Illumina, Sweden) primer pairs were used to add dual indices and Illumina sequencing adaptors to PCR amplicons generated in step one. PCR's were performed using KAPA hifi hot start polymerase ready mix (KAPA biosystems), purified using AMPure (Labplan) and

quantified using QUBIT dsDNA broad range assay system (Invitrogen Life Technologies) prior to pooling the amplicons at a concentration of 4nM each. The pooled library was denatured by addition of 0.2M NaOH followed by heating, diluted to 4pM and combined with an internal control for low diversity libraries (10% PhiX). The prepared pools were then sequenced on the MiSeq platform using the 300bp V3 kit reagents.

Bioinformatics

Compositional metagenomic 16S rRNA data was obtained for 62 samples (20 baseline, 4 samples 3 months after commencement of Ivacaftor (T3), 14 samples after 6 months of therapy (T6), 12 samples after 9 months (T9) and 12 samples after 12 months of treatment (T12)) in 300bp paired-end FASTQ format. Assembly of 300bp paired end reads was per-formed using FLASH (fast length adjustment of short reads to improve genome assemblies) (272). Quality filtering, including removal of mismatched barcodes and sequences failing the length requirement, was achieved using QIIME (Quantitative In-sights Into Microbial Ecology) (176). USEARCH v7 (64-bit) (178) was used for removal of background noise and chimeras, along with clustering of operational taxonomic units (OTUs). Alignment of OTUs was performed using PyNAST (Python Nearest Alignment Space Termination tool)followed by taxonomy assignment using BLAST against the SILVA SSURef database release 111 (176). Using phylogenetic trees created by PyNAST, alpha and beta diversities were calculated based on UNIFRAC distance matrices. Subsequent PCoA plots were visualised in EMPEROR v0.9.3-dev.

qPCR

Quantification of total faecal bacterial load of each subject was performed using qPCR. Samples and standards were analysed in triplicate using the Roche Lightcycler 480 platform and SYBR green reagent (Roche Diagnostics, UK). Primers, previously described, used were 5'-ACTCCTACGGGAGGCAGCAG-3' and 5'- ATTACCGCGGCTGCTGG-3' (273). A final concentration of 0.5 pmol of each primer was added to each reaction with a reaction cycle as follows: 95°C x 5 minutes, 45 cycles x 95°C 10 seconds, 60oC 30 seconds, 72°C 30 seconds, cooling for10 seconds. Mean concentrations were then expressed as $\log_{10}$ 16S rDNA copies per gram of faeces.

Statistical Analysis

Data was analysed using SPSS version 22.0 and STATA. Paired Wilcoxon signed rank test was used to analyse change in clinical parameters and inflammatory markers. Change in categorical variables was analysed using Chi squared test.

The Shannon diversity index (H) for the compositional microbiome data was calculated at the phylum, family, and genus levels as: $H = -\sum_{i=1}^{S} p_i \ln p_i$. Richness was calculated as the number of taxa present in each sample, and Evenness was calculated as H/log (Richness). The post-pre ivacaftor treatment differences for each of these measures were calculated for each patient. The means and 95% CIs for these differences were estimated via the bootstrap (with 1k replications).

Tests of post-pre ivacaftor treatment changes in specific phyla were based on centred-log-ratio (clr) transformations. First, the set of phyla was reduced to those present in all patients at both time points, and the percent of the total composition for each taxa (x_i) in this reduced set was calculated. The clr transformation for each phyla, for each time point, was then calculated as $clr(x_i) = \log(\frac{x_i}{g(x)})$, where g is the geometric mean. Linear mixed effects models (with a fixed effect of time and a random effect at the patient level) were then used to test pre vs post ivacaftor differences in the clr-transformed phyla.

Results

Fourteen patients participated, with a median duration of ivacaftor treatment of 12 months (range 3-12 months) at the time of post ivacaftor stool analysis. Table 5.3 summaries baseline characteristics. All patients had a class I-III mutation as their second mutation.

Table 5.3 Baseline Characteristics (n=14)	
Parameter	Median (Range)
Age	29 (18-39)
Gender	
Male	64.3%
Genotype	
G551D/F508del	78.58%
G551D/G551D	7.14%
G551D/R53X	7.14%
G551D/3028delA	7.14%
FEV_1 % predicted	65.5 (31-91)
BMI kg/m^2	22.05 (18.2-29.5)
Sweat chloride (mmol/l)	98 (81-155)

Clinical

Significant improvements in FEV_1 % predicted (*p<0.01*), BMI (*p<0.01*), sweat chloride (*p<0.01*) were observed after ivacaftor. Table 5.4 summaries change in clinical parameters. There was no statistically significant change in the CFQ-R eating domain (*p= 0.6*), or digestive Domain (*p=0.72*). A significant 64% reduction in intravenousantibiotics requirements was observed post treatment (*p<0.01*).

Table 5.4 Median clinical characteristics before and after ivacaftor (n=14)

	Pre ivacaftor	Post ivacaftor	*P value*
FEV_1 % predicted	65.5 (31-91)	72 (45 – 107)	*<0.01*
Sweat Chloride (mmol/l)	98 (81-155)	41.5 (24 – 93)	*<0.01*
BMI (kg/m^2)	22.05 (18.2-29.5)	23 (19.7 – 30.6)	*<0.01*
CFQ-R			
Eating	100 (77.8-100)	100 (77.8-100)	*0.6*
Digestive	88.9 (44.4-100)	88.9 (66.7-100)	*0.72*
Respiratory	66.7 (38.9-100)	88.9 (50-100)	*<0.01*

Gut inflammatory markers

No significant change in levels of biomarkers associated with inflammatory processes were observed between samples collected prior to commencing ivacaftor treatment when compared to samples collected POST-ivacaftor treatment (Figure 5.2). Levels of lactoferrin and calprotectin were observed to be similar between the two-sample time-points (p=0.27 and *p*=0.22, respectively) (Figure 5.2). All patients had a faecal elastase 1 level less than 100 µl/l before and after ivacaftor consistent with severe pancreatic insufficiency. There was no significant change in faecal calprotectin category (*p = 0.12*) (Figure 5.3).

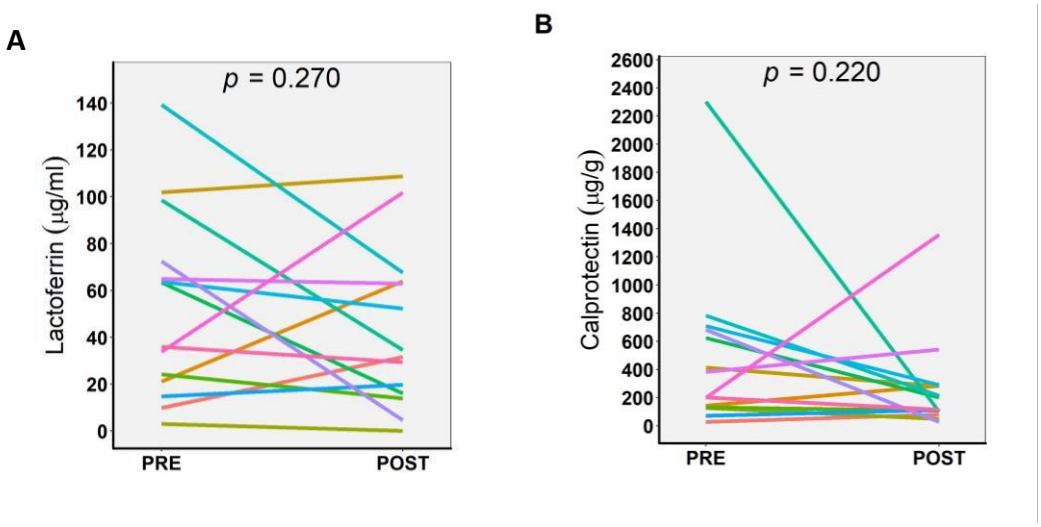
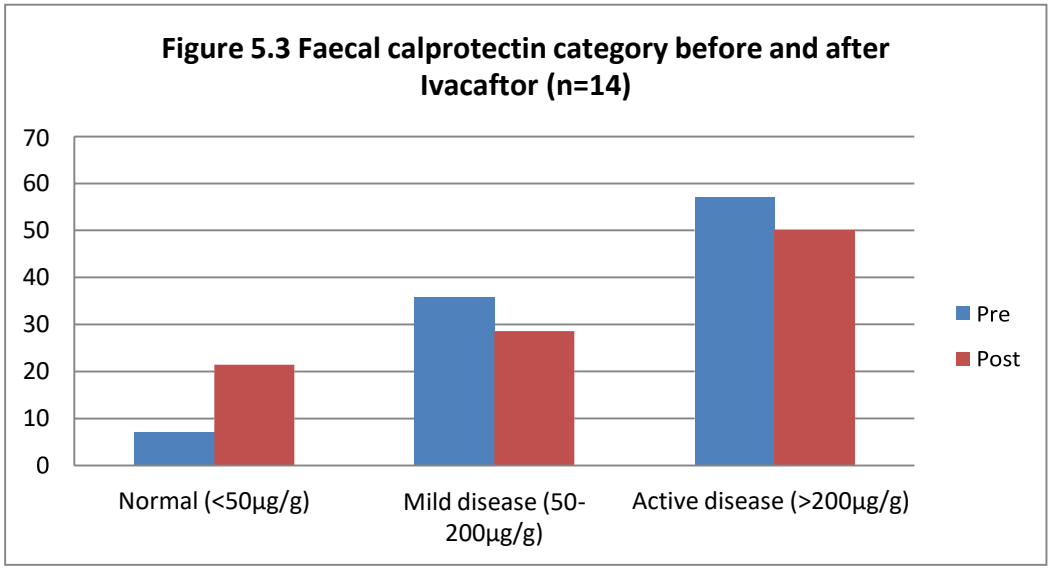


Figure 5.2 Faecal calprotectin & lactoferrin before and after ivacaftor (n=14)



Gut microbiota PRE- and POST-ivacaftor treatment

Following treatment with ivacaftor non-significant difference was observed in number of taxa. At the phylum level communities were predominately composed of *Bacteroidetes* and *Firmicutes*, with lower level of *Proteobacteria* detected in a number of samples (Figure 5.4 A). Overall, a marginal shift from higher levels of *Firmicutes* to

communities with higher abundance of *Bacteroidetes* were observed following ivacaftor treatment (Figure 5.4 A and 5.4B) (*p=0.04* and *p=0.05*), however this shift wasstatistically non-significant following adjustment for multiple testing.

In total, we detected 50 different bacterial families at the combined visit time-points (total PRE: 48 [range 21-32]; total POST: 46 [range 20-31]) (Figure 5.4C). Amongst taxa that accounted for >1% of total read number at the family level, thirteen out of the total of fourteen were shared between PRE- and POST-ivacaftor treatment visits (Table 5.5). Only two bacterial families were observed to be significantly different between the PRE- and POST-ivacaftor visits (Table 5.5). However, neither *Bacteroidaceae* nor *Porphyromonadaceae* remained significantly different following an adjustment for multiple testing (*p>0.05*).

Following treatment with ivacaftor, there was no significant change in the main ecological community indices such as taxonomic richness [S], Shannon-Wiener diversity [H'], evenness [$e^{H/S}$] or dominance [D] when compared to PRE-ivacaftor samples (Figure 5.5).

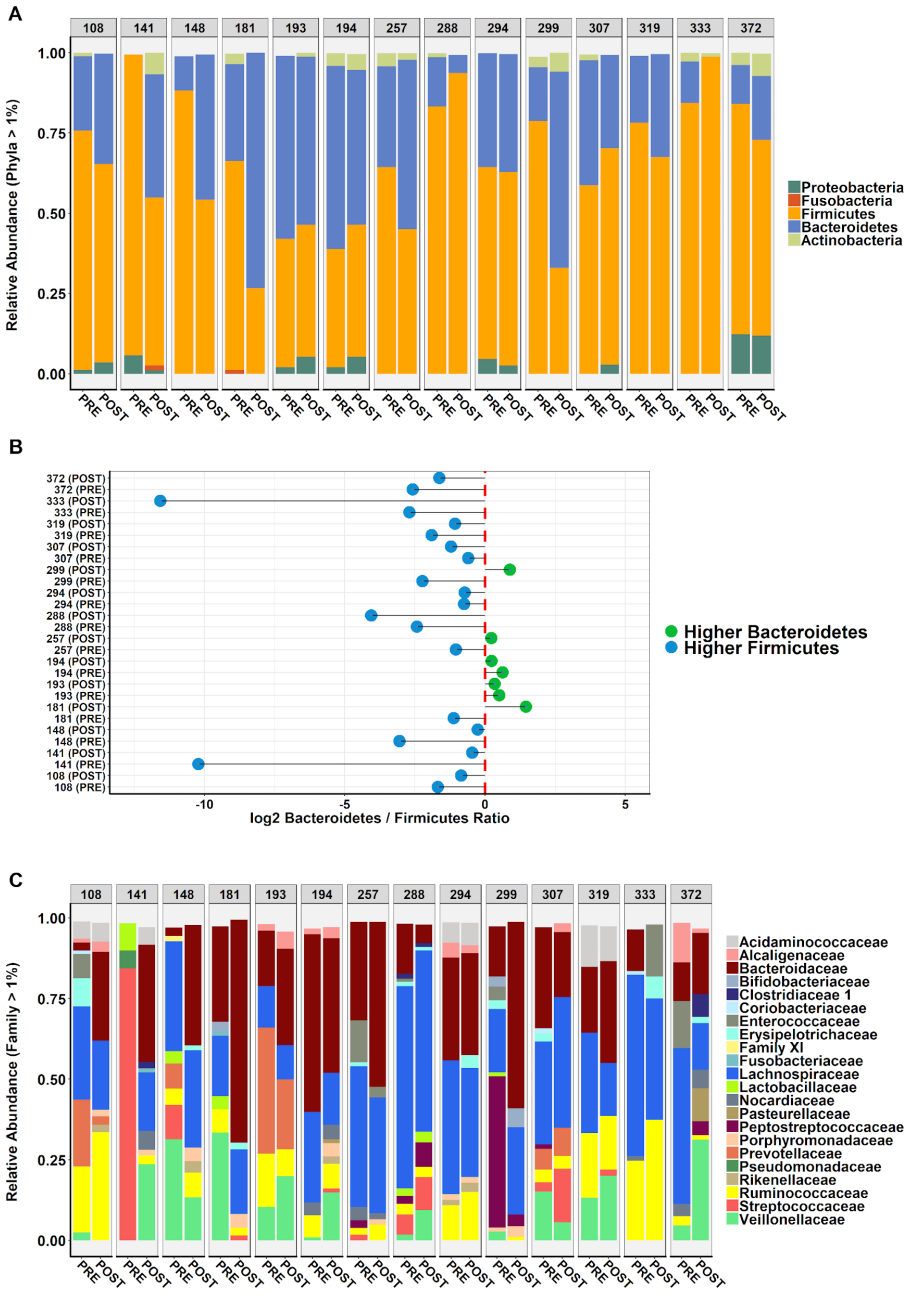


Figure 5.4. (A) Relative abundance (%) for the main phyla PRE- and POST-ivacaftor treatment, (B) Firmicutes/Bacteroidetes ratio (log2-fold change) between PRE- and POST-ivacaftor treatment, (C) Relative abundance (%) of families between PRE- and POST-ivacaftor treatment for each individual. Values shown depict percentage relative abundance >1% of the totalbacteria detected (n=14).

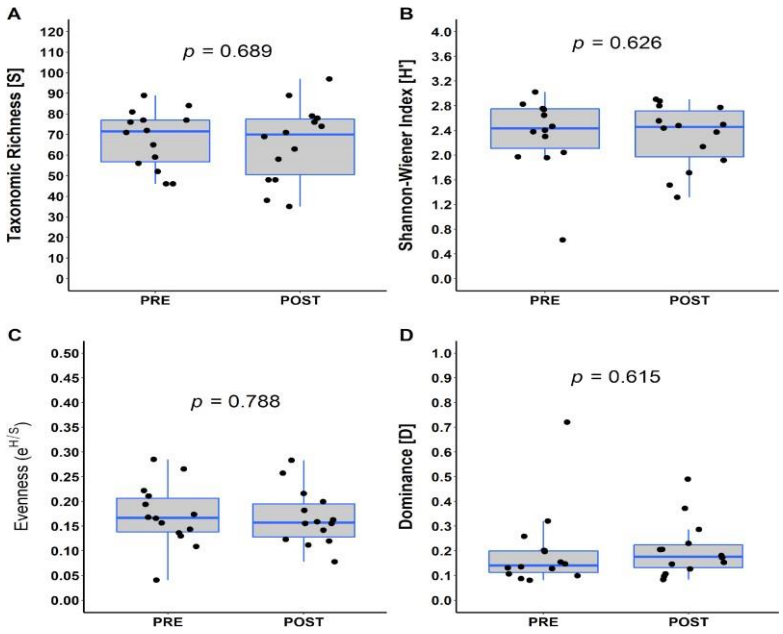


Figure 5.5 Changes in ecological indices following treatment with ivacaftor. Comparison between PRE- and POST-ivacaftor treatment visits; (A) taxonomic richness [S], (B) community diversity (Shannon Wiener Index [H']), (C) community evenness [$e^{H/S}$] and taxonomicdominance [D]. P<0.05 denotes statistical significance (n=14).

Microbial variance explained by clinical and other covariates

Ordination using principle coordinate analysis (PCoA) of CLR-transformed OTU counts showed that there were no clear clusters of samples based on sample groups (PRE- vs. POST-ivacaftor) (Figure 5.6A). Furthermore, at the level of family limited shifts in relative abundance were observed, with only *Bacteroidaceae* and *Porphyromonadaceae* demonstrating difference between samples groups (*p=0.05* and *p=0.04*, respectively) (Figure 5.6B and Table 5.5). However, those differences were non-significant following adjustment for multiple testing (*p>0.05*). Furthermore, we employed Bray-Curtis dissimilarity measurements between pre- and post-ivacaftor

treatment time-points (beta-diversity) in order to identify and compare the underlying bacterial community structures. Dispersion using the permuted betadisper test, as implement within the vegan package [ver. 2.5-4] (274) was non-significant between PRE- and POST-ivacaftor visits (*p=0.26*) (Figure 5.6A).

Table 5.5 Change in gut microbiota after ivacaftor (n=14)

Family	PRE (Mean RA; Range)	POST (Mean RA; Range)	*p-value*
Bacteroidaceae	19.82 (0.06-55.21)	32.81 (0.02-69.08)	***0.046‡***
Lachnospiraceae	32.69 (0.07-62.73)	27.00 (10.67-56.26)	*0.119*
Ruminococcaceae	8.89 (0.05-24.71)	10.44 (1.17-37.46)	*0.463*
Veillonellaceae	8.35 (0-33.535)	9.89 (0-31.28)	*0.715*
Streptococcaceae	7.79 (0.04-84.32)	2.45 (0.01-16.68)	*1.000*
Prevotellaceae	5.32 (0-39.10)	2.44 (0-21.70)	*0.834*
Acidaminococcaceae	1.72 (0-12.70)	2.07 (0-10.72)	*0.894*
Porphyromonadaceae	0.50 (0-1.83)	1.72 (0-4.23)	***0.036‡***
Enterococcaceae	3.03 (0-14.66)	1.52 (0-16.04)	*0.142*
Erysipelotrichaceae	1.39 (0.04-8.81)	1.45 (0.09-6.95)	*0.808*
Alcaligenaceae	1.60 (0-12.24)	1.33 (0-5.24)	*0.906*
Nocardiaceae	1.06 (0-4.00)	1.30 (0-5.98)	*0.529*
Peptostreptococcaceae	3.91 (0.04-46.96)	1.27 (0.01-7.60)	*0.952*
Lactobacillaceae	1.53 (0-8.34)	0.35 (0-3.25)	*0.124*

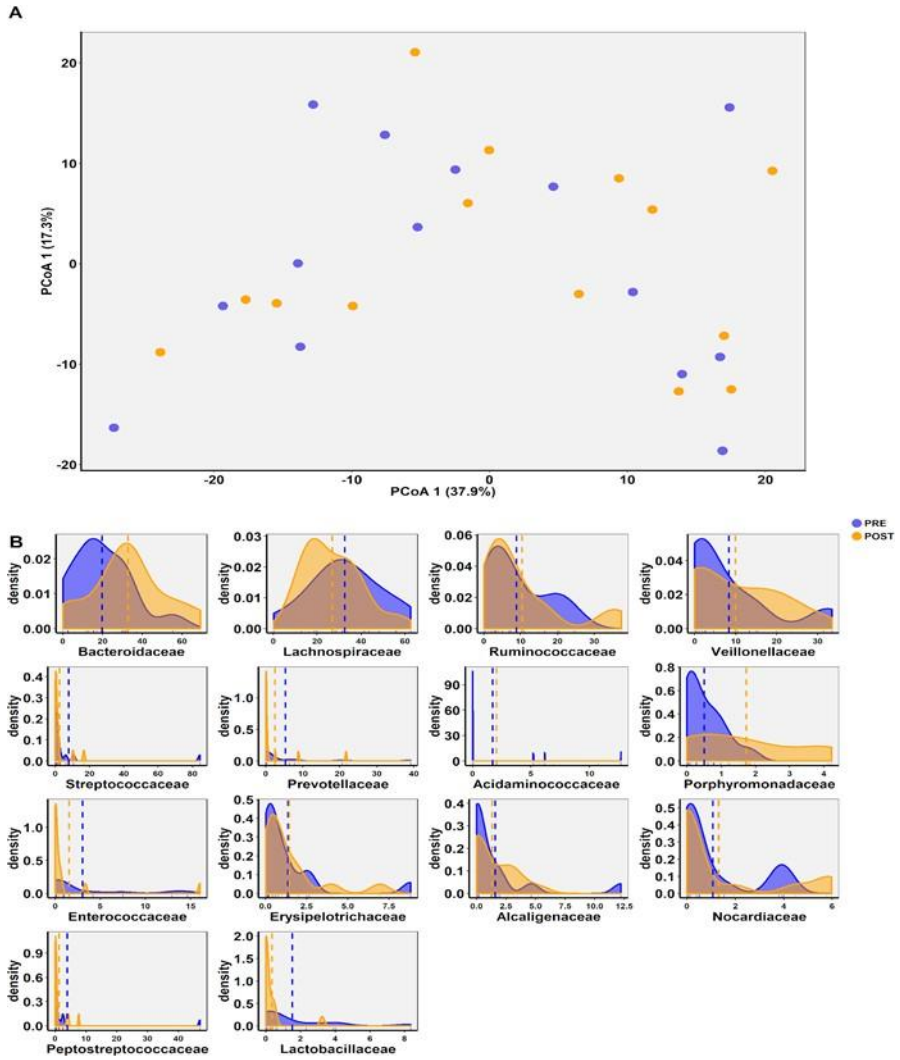


Figure 5.6 (A) principal coordinate analysis on clr-transformed OTU data and Euclidean distance for Pre and Post ivacaftor treatment visit. (B) Distributions of the relative abundances (%) for the top taxa (family; >1% total abundance) between Pre (blue) and post ivacaftor (orange) treatment visits. Dashed lines denote spread of the mean for relative abundances (n=14).

Discussion

In a clinically responsive cohort, we did not observe a significant change in markers of gut inflammation after ivacaftor. Given that potentiation of CFTR function did not result in a reduction in gut inflammation this would suggest that additional

mechanisms give rise to this process in CF. Previous work in children with CF demonstrated a reduction in faecal calprotectin levels after treatment with probiotic in the form of *Lactobacillus rhamnosus* (268). It has been suggested that there may be a relationship between the intestinal microbiota composition and inflammation. The lack of a significant change in the gut microbiota in this study would be in keeping with this hypothesis in CF. A small study in ten patients treated with ivacaftor demonstrated an improvement in gastrointestinal pH but no change in gastro-intestinal motility with restoration of CFTR function (275). However, despite the ability of ivacaftor to improve gut pH no effect was noted on inflammation.

In contrast to probiotic treatment in CF which has resulted in significant improvements in gut dysbiosis and increase in gut diversity with treatment ivacaftor did not result in a significant improvement in gut microbiota despite a significant reduction in IV antibiotics requirements (276). This may reflect a multi-factorial aetiology of alterations in the gut microbiota in CF. Previous work has demonstrated a reduction in the relative abundance of *Bacteroidetes* and increase in *Firmicutes* and in adults with CF compared to a control cohort at a phylum level (277). Interestingly in that work patients with CF with the highest intravenous antibiotic requirements had the lowest *Bacteroidetes* and highest *Firmicutes* (277). We observed an increase in *Bacteroidetes* and reduction in *Firmicutes* after ivacaftor treatment which was during a period in which IV antibiotic consumption decreased by 75% and hence this may representreduced antibiotic usage.

In contrast to our results a recent study evaluating changes in the gut microbiota in 16 patients with CF (8 adults and 8 children) after ivacaftor demonstrated a significant reduction in faecal calprotectin (278). This contrast may reflect our smaller sample size of fourteen. It is also possible that age played a role in these differences. The median age of the cohort reported by Ooi et al. was 17.1 years, while the median age of our cohort was a decade older at 29 years. Hence, it may be that age or more severe disease plays a role in these findings. In keeping with the findings of Ooi et al. we did not find a significant change in alpha or beta diversity (278).

Conclusion

There was no significant change in gut inflammation or gut microbiota after treatment. A longer duration of treatment may be required to observe significant change. It is possible the older age and more severe disease may play a role in this lack of change.

Limitations

A limitation of this study is the small sample size of 14 and the lack of a control cohort. However, given that the global prevalence of the G551D mutation is 4-5% this represents a relatively large single centre sample size. The Ooi et al. study was a multi- centre study and noted differences in the gut microbiota across the participating sites and postulated that this was environmental related, thus the single centre nature of this study negates this limitation. An additional limitation is that it is predominantly an adult, cohort. It is possible that treatment in younger children with a shorter duration of pancreatic insufficiency may result in some restoration of exocrine pancreatic function after ivacaftor.

Chapter 6

Precision medicine and predicting response to CFTR modulation

Introduction

It is estimated that on a daily basis millions of patients worldwide take a medication that will not confer benefit to them as an individual, with estimates from the United States suggesting that the percentage of patients who actually benefit from the top tenhighest earning drugs ranges from 4 to 25% (279). For example, estimates suggest that40% of asthmatic patients will not experience a clinical improvement from the initial treatment which they are prescribed. In the case of statins, it has been suggested that as few as 2% of patients may actually benefit from them (280, 281). It has beenpostulated that genetic variations in genes encoding enzyme responsible for drug metabolism may be among that factors which contribute to this (282-284). It has previously been demonstrated that patients with asthma who are homozygous for the B_2-Arg-16 mutation experienced a worsening of asthma– as reflected by a decline in peak expiratory flow - with regular albuterol (salbutamol) use as opposed to as needed use. The recognition that many factors including genetic, environmental and dietary factors may play a role in treatment response has stoked renewed interest in the concept of precision medicine.

Precision medicine

The notion of precision or individualised medicine, involves treating individual patients while taking individual variability into account is not a new concept. The advances in the ability to characterise individual patients based on proteomics, metabolomics, genomics, and even mobile health technology has enhanced the potential to apply this

concept more broadly (285). While randomised controlled trials are regarded by healthcare professionals as the gold standard evidence base for the utility of a medication in informing patient treatment it must be recognised that this type of trial provides an insight into the average benefit of treatment in all patients as opposed to an assessment of the benefit which it may confer to any individual patient. It some contexts it can be difficult to apply the results of any given clinical trial to an individual patient as they may not be similar to the patients included in the clinical trial. This is of importance in cystic fibrosis given that patients with an FEV_1 less than 40% predicted are frequently excluded from clinical trials, despite the fact that they are the cohort who may potentially benefit most from new treatments. N-of-1 trials involve conducting multiple typically blinded and randomised crossover trials in a single patient. In this type of study, the participant changes from placebo to active treatment at intervals (286-288). They may be useful in situations where the comparative effectiveness of a medication being considered for an individual patient is unclear and where the existing clinical trial data may not apply to the individual patient being treated (289).

If the desire is to conduct a series of n-of-1 trials the same outcome measure is used in each patient, however, given that each patient in an n-of-1 study is analysed individually there is the potential to utilise different patient specific endpoint or outcome measures to assess each patients treatment response (290). Personalised or precision medicine aims to identify the treatment with the greatest likelihood of conferring benefit to the individual patient by considering patient specific factors (291).

In a real-world clinical context at the individual level clinicians practice precision medicine by trial and error changing therapy in individual patients to achieve optimal results.

Thus, in an era of precision medicine this chapter presents a series of case reports illustrating the benefit of CFTR modulation in patients who would not have met drug study inclusion criteria and clinical benefits which were not primary endpoints in clinical trials but are clinically meaningful to the individual patient. This is followed by an evaluation of the relationship between forskolin induced organoid swelling with ivacaftor and the change in FEV_1 in a cohort of patients with the G551D mutation to explore the potential for predicting clinical response with an in vivo model.

Case 1: The Role of Ivacaftor in Severe Cystic Fibrosis in a patient with the R117H mutation

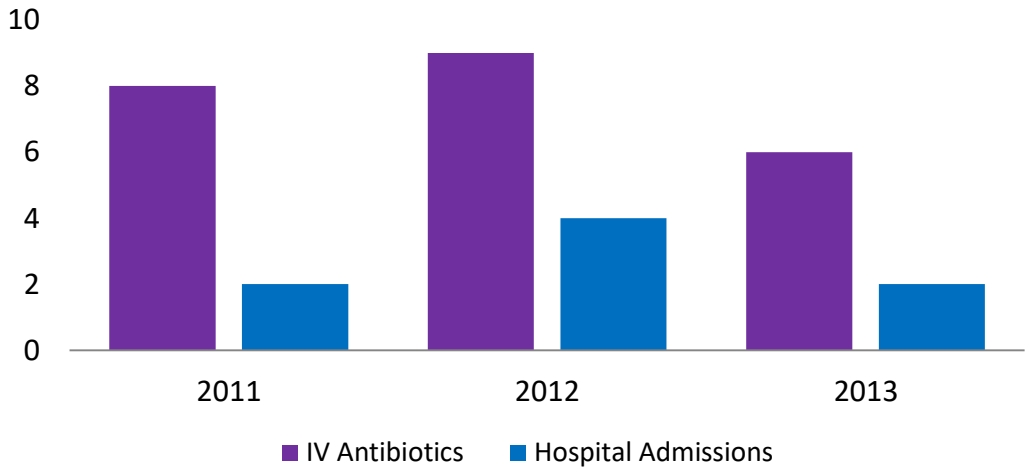
Introduction:

As discussed in previous chapters the benefit of ivacaftor in patients with CF with Class III (gating) is well established. The R117H mutation is a class IV (conductance) mutation in which CFTR is correctly localised at the cell surface but conducts chloride abnormally. Clinical trial data demonstrate 24 weeks of Ivacaftor treatment produces a5% mean absolute increase in FEV_1 , 0.31 kg/m^2 mean absolute increase in BMI, -21.9 mmol/L mean absolute reduction in sweat chloride and 12.6 point absolute increase in CFQ-R respiratory domain in patients aged 18 and older who carry the R117H mutation (37, 38). However, this study excluded patients with severe disease (FEV_1 < 40% predicted).

Case Presentation: BD is a 56-year-old lady with severe CF (F508del/R117H- 5T); with a baseline FEV_1 of 28% predicted and a high exacerbation rate – requiring 8 courses of IV antibiotics in 2011(2 in-patient), 9 courses of IV antibiotics in 2012 (4 in-patient) – despite optimisation of her medical therapy. She had a bronchoscopy and BAL performed which grew *Stenotrophomonas maltophilia*, with no evidence of non-tuberculous mycobacteria (NTM), *Burkholderia cepacia complex*, fungus or other CF associated pathogens. Treatment of her gastro-oesophageal reflux disease was optimised, initially with twice daily proton pump inhibitor and subsequently a Nissen's fundoplication. She did not have any evidence of allergic bronchopulmonary

aspergillosis (ABPA) or CFRD. She commenced nebulised Aztreonam (Cayston™) with a subsequent reduction in her IV antibiotic requirements to 6 courses (2 in-patient) in 2013 (Figure 6.1).

Figure 6.1 Antibiotic requirements & hospitalisations



She was considered an unsuitable candidate for lung transplantation due to a combination of her microbiology – pan-resistant Stenotrophomonas maltophilia – and chest wall deformity due to extensive and disproportionate left lung bronchiectatic destruction. In February 2014 she commenced ivacaftor on a named patient basis (compassionate use programme). Baseline spirometry, weight, sweat test and Cystic Fibrosis Questionnaire Revised (CFQ-R) (122, 123) were recorded before commencing ivacaftor. High resolution CT Thorax (HRCT) was performed using an ultra-low dose protocol (171) at baseline and after 3 and 6 months of treatment (mean effective radiation dose 0.08 mSv). Blood samples were obtained at baseline and after 3 and 6

months of treatment and analysed for circulating cytokines using an ELISA on an MSD (Mesoscale Discovery) platform (229, 230). Table 6.1 summarizes treatment response.

Table 6.1 Change in clinical parameters after ivacaftor

Characteristic	Baseline	3 Months	6 Months	Δ 6 Months
FEV_1 (%)	28	28	28	0
Weight (Kg)	40	43.4	45.8	↑ 5.8
BMI (Kg/m^2)	15.8	17.2	18.1	↑ 2.3
Sweat test (mmol/l)	68	24	40	↓ 28
6 Minute Walk test				
Distance (metres)	330	320	360	↑ 30
Borg Score at 6 minutes	3	3	2	↓1
SaO_2 at 6 minutes	91%	91%	93%	↑ 2
CFQ-R Domain				
Respiratory	44.4	66.7	77.8	↑ 33.4
Physical	4.2	45.8	58.3	↑ 54.1
Emotion	60	80	86.7	↑ 26.7
Vitality	8.3	75	58.3	↑ 50
Eating	0	88.9	88.9	↑ 88.9
Health Perception	33.3	66.7	66.7	↑ 33.4
Body Image	33.3	66.7	100	↑ 66.7
Weight	0	33.3	100	↑ 100
Digestive	88.9	88.9	88.9	0

At 6 months FEV_1 remained stable, weight increased by 5.8 kg, and sweat chloride fell by 28 mmol/l. In the six months after commencement of Ivacaftor she had one course of Intravenous (IV) antibiotics (0 inpatient days) in contrast to four courses of IV antibiotics (19 inpatient days) in the corresponding six-month period in the previous year. Subjectively she discontinued her home oxygen for routine activities after 3weeks of treatment. Quality of life improved across all domains of her CFQ-R afterthree and six months of Ivacaftor (Table 6.1). Figure 6.2 demonstrates the change in

circulating cytokine profile at baseline and after three and six months of Ivacaftor, with an increase in IL- 6 and IL-8.

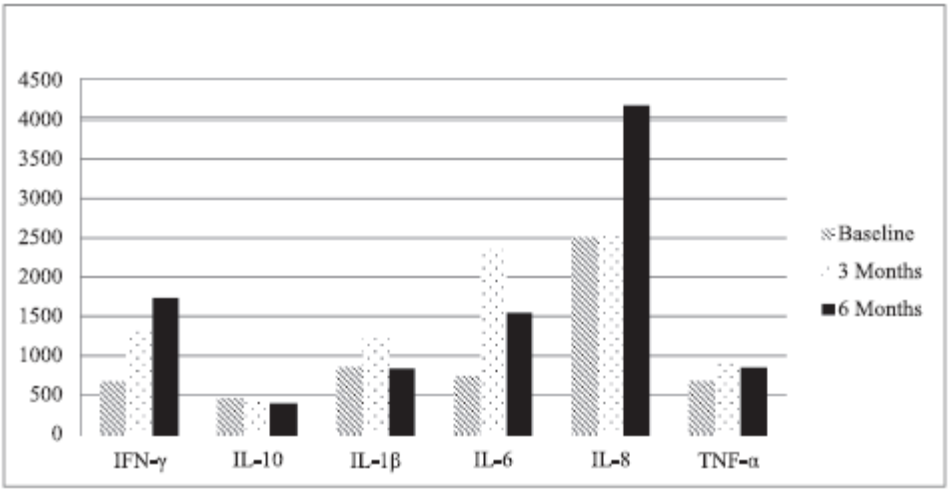


Figure 6.2 Change in circulating blood cytokines before and after ivacaftor

HRCT demonstrated a reduction in mucus plugging and in bronchial wall thickening in the functional right lung after 6 months of treatment (Figure 6.3A, B, and C).

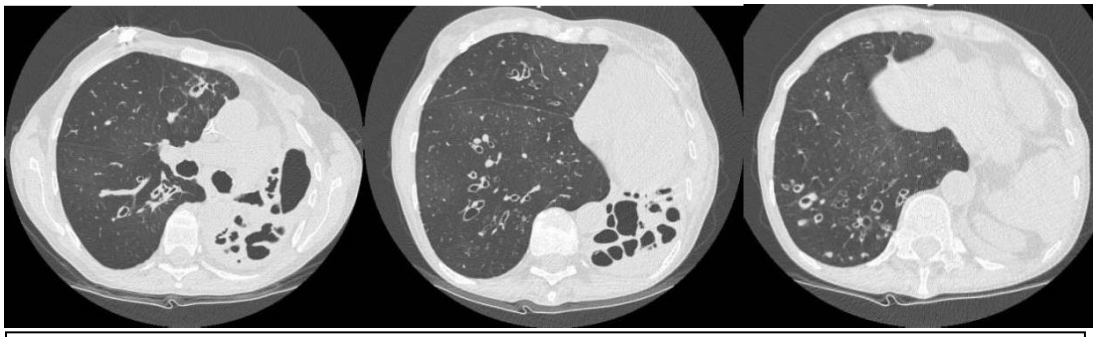


Figure 6.3A Baseline Axial HRCT shows bronchiectasis with bronchial wall thickening in focal areas, throughout the right lung. There is severe volume loss with bronchiectasis and parenchymal lung destruction on the left side.

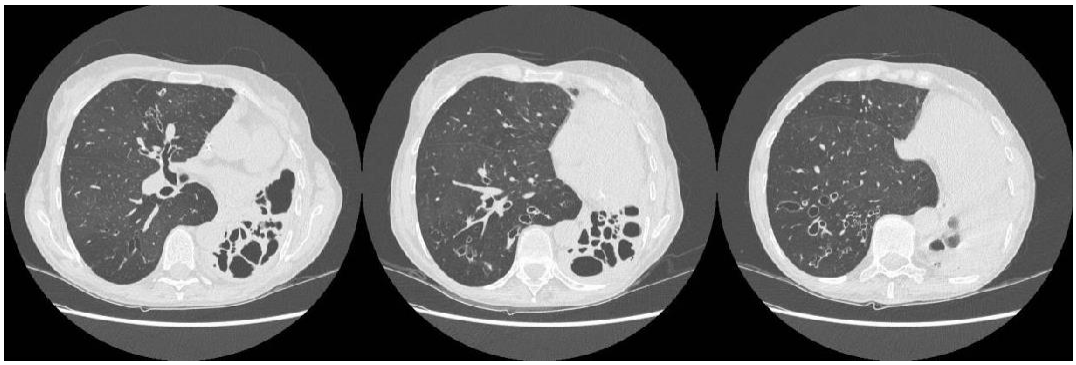


Image 6.3B Axial HRCT after three months of Ivacaftor shows interval improvement in bronchial wall thickening in all the focal areas of bronchiectasis seen on pre-treatment studies. There is no change was found in the left hemi-thorax.

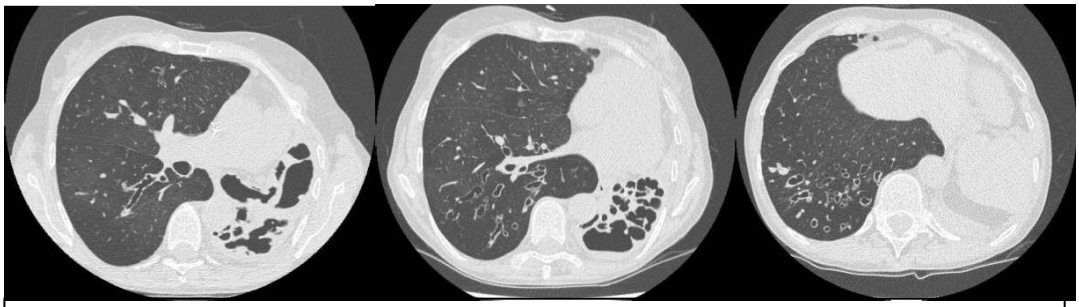
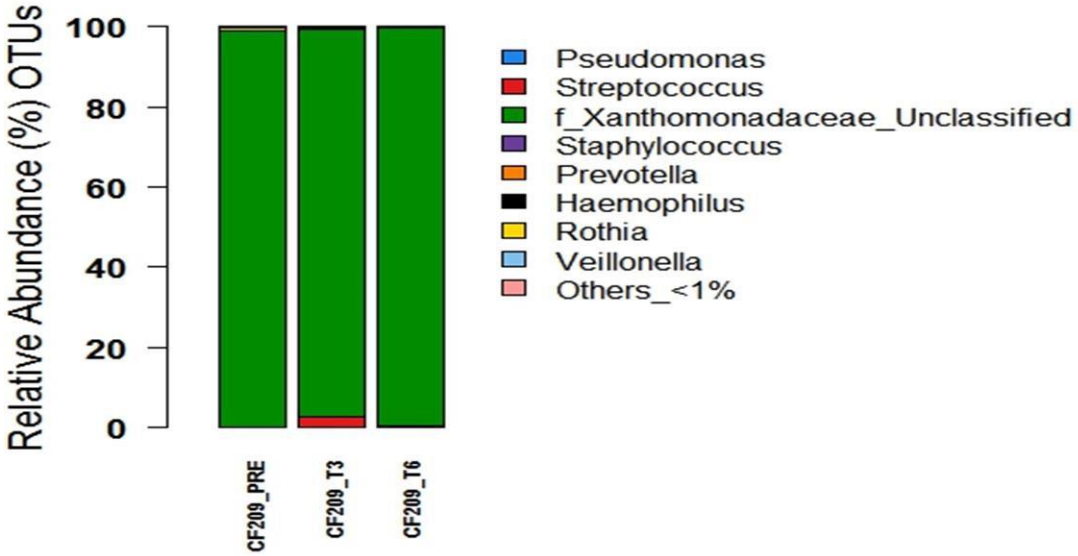


Image 6.3C Axial HRCT after six months of Ivacaftor shows stable appearances when compared to the 3-month study. Once again, no change was found in the left hemi-thorax.

There was no change in her sputum microbiome after ivacaftor therapy which continued to grow *Stenotrophomonas maltophilia (Xanthomonadaceae_unclassified)* as the predominant organism (Figure 6.4).

Figure 6.4 Sputum 16s sequencing before and after ivacaftor



Discussion: This single patient case demonstrates the utility of ivacaftor in a patient with severe CF and the R117H mutation. Improvement in FEV_1 is frequently a primary end point in clinical trials assessing new therapies in CF. Based on change in FEV_1 this lady would be considered a treatment failure; however, her quality of life, weight, exacerbation rate and imaging all improved; highlighting that in severe disease traditional markers of treatment response may not be a true reflection of therapeutic benefit.

Recurrent pulmonary exacerbations are associated with more rapid decline in FEV_1 (113, 114). Her pulmonary exacerbation frequency decreased on ivacaftor. A significant

reduction in pulmonary exacerbations was not observed in the Konduct study cohort who had less severe disease and this reduction may be unique to a cohort with more severe disease hence highlighting the utility of case studies in this patient cohort. Given that she is not a candidate for lung transplantation, prevention of exacerbations and resultant decline in FEV_1 could be important in on-going management. Accordingly, ivacaftor may have a role in deferring the need for transplantation or as an alternative therapy in patients who are not suitable for transplantation in this cohort.

An increase in all domains of her CFQ-R was observed, which has not been observed in studies of ivacaftor in patients with gating mutations or residual function mutations and, therefore, may be unique to patients with severe CF and the R117H mutation. The increase could also reflect further benefit in the setting of severe CF or be partially due to ascertainment bias (due to lack of blinding) and further reflects the utility of using n-of-1 studies to assess improvement in multiple clinical parameters in individual patients. Paradoxically, pro-inflammatory cytokines increased after commencingivacaftor during a period when she had fewer pulmonary exacerbations. This change may represent a diminution of immunoparesis (292, 293) or be related to her increased exercise (294). The radiological changes mirror those seen following ivacaftoradministration in other gating mutations (77, 78) and may reflect an enhancement of mucociliary clearance and improved airway surface liquid with ivacaftor therapy as described in earlier chapters. There was no change in her lung microbiome either on 16s sequencing or on standard laboratory culture which continued to grow pan resistant *Stenotrophomonas maltophilia*.

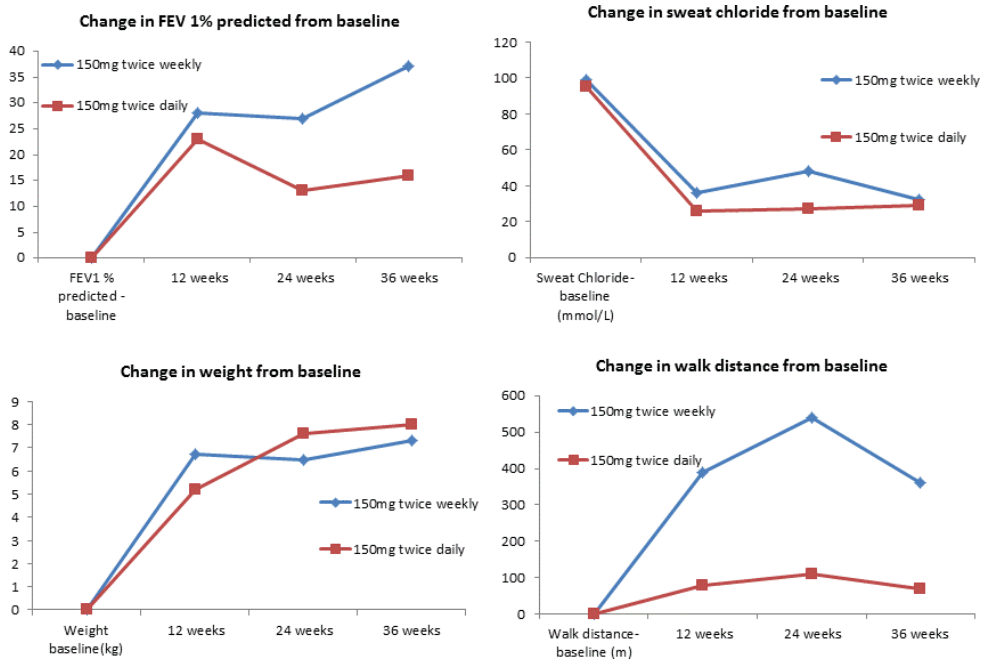
Case 2: Ivacaftor therapy in siblings with cystic fibrosis- the potential implications of Itraconazole in dosage and efficacy

Introduction: As discussed in the real world clinical effectiveness of an intervention may be poorly demonstrated by a clinical trial as patient cohorts are often selected based on strict criteria (295). Recent published data demonstrate the clinical effectiveness of ivacaftor in patients with CF with an FEV_1 less than 40% predicted (282, 283) and in patients with gating mutations other than G551D (36). These groups had been excluded from the Phase III clinical trials and as a result data regarding the use of ivacaftor in these subsets were not previously available to clinicians. Furthermore, important adverse events including drug-drug interactions may not occur within the relatively small sample size of a clinical trial (295). Ivacaftor is extensively metabolised in the liver by the CYP3A enzyme. Dosage adjustments from the standard dose of 150mg twice daily are necessary if ivacaftor is administered with moderate or strong CYP3A inhibitors, with reductions to 150 mg once daily and 150 mg twice weekly, respectively. There are no published data regarding the effect of CYP3A inhibitors on dose of ivacaftor therapy in patients with CF.

Case: We report Ivacaftor usage in siblings who were both heterozygous for the G551D mutation. A 30-year-old female (pre-treatment FEV_1 59% predicted) was commenced on the standard dose of Ivacaftor, 150mg twice daily, in March 2013. Her 20-year-old brother (pre-treatment FEV_1 47% predicted) was previously diagnosed with allergic bronchopulmonary aspergillosis (ABPA) and was receiving ongoing treatment with itraconazole (200mg once daily) and had his ivacaftor dose adjusted according to

manufacturer's guideline to 150mg twice weekly. Despite the significant dose reduction in one sibling, similar improvements were seen in lung function, sweat chloride level and weight over 36 weeks (Figure 6.5). Similar changes in total Bhalla score for CT Thorax were also observed in both. Interestingly, there was an increased improvement seen in modified shuttle walk test in the patient receiving ivacaftortwice-weekly.

Figure 6.5 Change in clinical parameters in siblings commenced on ivacaftor



Discussion: Deliberate use of combinations of drugs utilising CYP3A4 inhibition to improve efficacy and/ or reduce cost is reported with other therapies, e.g. immunosuppressant and anti-retroviral therapies (296-298) . Itraconazole is a commonly used drug in CF, which is widely available and relatively inexpensive, and

has previously been reported to cause significant non-beneficial drug-drug interactions due to CYP3A inhibition (299). Given that these co-habiting siblings had shared dietary and other environmental exposures, concomitant use of Itraconazole with Ivacaftor in one sibling appears to explain the similar outcomes during 36 weeks of ivacaftor therapy despite that sibling using only 1/7th the standard dose. This case highlights the importance of understanding drug-drug interactions to avoid potential toxicity when introducing new therapies. It also specifically highlights the need for the clinician to be aware of the importance of reducing the dose of ivacaftor when used concomitantly with CY3PA inhibitors. However, caution is required when attempting to extrapolate from a single case report to a population effect, especially as the effect of Itraconazole may vary amongst individuals as absorption of itraconazole varies according to the formulation used, fasting status of the patient, or concomitant administration of proton pump inhibitors or H_2 receptor antagonists (300) . Equally in a recent small study which evaluated ivacaftor drug levels in patients taking the standard dose of ivacaftor (150mg twice daily) considerable variability in ivacaftor drug levels from 400- 3000ng/ml (301) . The study participants did not have hepatic impairment and were not taking medications which inhibit CYP3 and yet 5 of the 6 participants had ivacaftor levels which were considerably higher than those observed in clinical trials. When compared with his sibling, the clinical improvements seen during reduced-dose Ivacaftor therapy in our case suggest that the reduced dose prescribed was sufficient to achieve clinical effectiveness. This case is unique in that the response to reduced- dose ivacaftor in the 20-year-old male can be benchmarked against the response seen

in his sibling on a standard dosing regimen; however, most clinical cases lack this familial comparison. Routine drug level testing for ivacaftor is currently not available. In its absence we recommend close clinical monitoring along with monitoring of sweat chloride response, although controversial (101, 302), to ensure maximum clinical effectiveness. This is the first case to highlight this potentially common drug-drug interaction in CF, and raises important issues surrounding the use of ivacaftor in the clinical setting and the need to introduce access to standard drug level testing for clinicians using this personalized medicine.

Case 3: Partial restoration of exocrine pancreatic function in an older child with Cystic Fibrosis

Introduction: The long-term utility and respiratory benefits (as reflected by an increase in FEV_1 and reduction in pulmonary exacerbations) of ivacaftor in patients aged 6 and older with cystic fibrosis (CF) with the G551D mutation are well established (22). Among the comorbidities observed over time in CF gastrointestinal symptoms and exocrine pancreatic dysfunction are prominent features; with exocrine pancreatic insufficiency (PI) traditionally being regarded as irreversible on serial testing (303).Faecal elastase 1 (FE-1) is a glycoprotein which is synthesised in the pancreas and excreted in the stool. It is not degraded on passage through the GIT and consequently considered a sensitive marker of exocrine pancreatic function; with levels <200 μg/g stool being considered indicative of pancreatic insufficiency.

The KIWI study which highlighted the safety and efficacy of ivacaftor in children aged 2-5 with CF with gating mutations (25). At baseline 93% of children had a FE-1 less than 50 μg/g consistent with severe pancreatic insufficiency. Notably a 99.8 μg/g significant mean increase in FE-1 was observed after 24 weeks of treatment. Equally of interest was the observation that 26% of children had an FE-1 above the threshold of 200 μg/g considered indicative of PS at least once over the course of the study. This raises the biologically plausible possibility that commencement of CFTR modulatory in very young children may augment exocrine pancreatic function. However, whether this is also the case in older children and adults with CF is unclear.

We recently observed dynamic changes in FE-1 in an older child (age 6·5 years) after commencing ivacaftor. We present the case of a female (genotype F508del/G551D) diagnosed at four years and nine months, after hospital admission with increased respiratory effort and cough. FE-1 was measured at diagnosis and consistent with severe pancreatic insufficiency at <15 μg/g. At this point the patient was commenced on four Creon™ 10,000 capsules per day. The patient started Ivacaftor therapy on her 6th birthday in keeping with local regulatory guidelines at the time.

Figure 6.5 demonstrates the change in FEV_1% predicted and BMI Z scores after ivacaftor. After six months of ivacaftor she reported abdominal pain and constipation. As a result, her enzymes were reduced by 50% to a total of 2 Creon™ 10,000 capsules per day. At this point FE-1 was measured and within the normal range at 259 μg/g. Twosubsequent FE-1 levels after seven months of treatment were 118μg/g, and 121μg/g consistent with moderate pancreatic insufficiency (Figure 6.6).

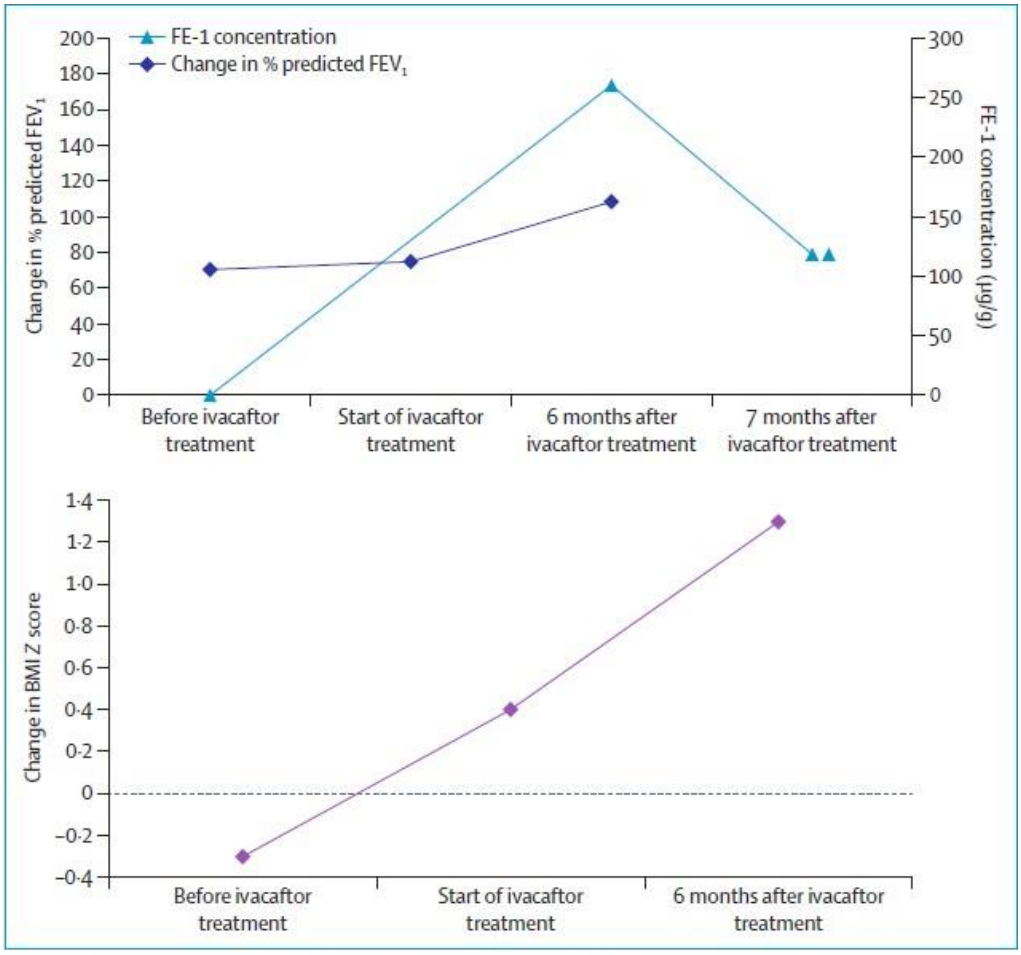


Figure 6.6 Change in FEV_1% predicted, FE-1 and BMI Z-score after ivacaftor

The KIWI study highlighted the potential for a partial restoration of pancreatic function in early life in some of those treated with ivacaftor (25). We concur with this assessment. However, until the potential impact of these therapies has been established in all patients commencing CFTR modulatory therapy serial monitoring of exocrine pancreatic function with FE-1 levels in all age groups. While in there are case reports in adults receiving ivacaftor experiencing a reduction in pancreatic enzyme replacement requirements after treatment, in chapter 4 we did not observe any significant change

in faecal elastase levels in a adult cohort after initiation of ivacaftor (84). Thus, it may be that older age and disease severity play a role.

Case series 4: The relationship between forskolin induced organoid swelling and change in FEV_1 after ivacaftor

Introduction

The intestinal tract develops from the same embryonic germ layer as the lung. Given that CFTR is expressed in the gut a number of gastrointestinal modalities may potentially be useful in establishing a diagnosis of CF, assessing CFTR function and predicting treatment response. Intestinal current measurement (ICM) can be utilised asan ex-vivo method to evaluate CFTR function in rectal suction biopsy samples (304). In this method the transepithelial short circuit current is measured by adding various chemical baths which stimulate ion secretion to the rectal biopsy sample in a mini- Ussing chambers (305). The trans epithelial short circuit current is a surrogate measurement of ion transport related to chloride transport. It has been used to aid in establishing a diagnosis in patients with atypical CF (304). A small study demonstrated a significant increase in in cAMP mediated transepithelial short circuit current after ivacaftor, to a level equal to approximately 51% of that observed in age-matched healthy controls (306).

A novel method for assessing CFTR function using a primary intestinal culture model termed organoid has been developed. An organoid is a miniature and less complex three-dimensional (3D) representation of an organ. It retains the anatomical and functional characteristic of the organ of origin. Intestinal organoids may be produced from rectal biopsy samples and can be cultured for long term use by treating them with

a variety of growth factors (307-309). The resultant organoids develop resulting in the production of a mini gut containing all major epithelial cell types and villus and crypt like structures.

CFTR plays an important role in electrolyte and fluid transport in the lungs and the gastrointestinal tract. Forskolin increases cAMP levels which in turn gives rise to robust CFTR activation. This results in fluid secretion into the organoid lumen and subsequent organoid swelling. This forskolin induced organoid swelling can be assessed in an unbiased manner using an automated fluorescent image analysis (105, 310). Forskolin induced organoid swelling is entirely CFTR mediated as reflected by comparing health human organoids to CFTR deficient or CF organoids (106, 310). The degree of organoid swelling can be quantified by staining the organoids with calcein green and making use of a confocal microscope 1 hour after treatment with forskolin (310). The swelling is then represented numerically as the measurement of the total green surface area in allthe organoids in an individual well can comprising it to baseline observations. Importantly this forskolin induced organoid swelling can be modulated by CFTR inhibitors and CFTR modulators including ivacaftor (311, 312).

FIS has been shown to correlate with disease severity. In a study evaluating 34 infants with cystic fibrosis, children with lower FIS were observed to have significantly higher levels of Immunoreactive trypsinogen and were observed to be more likely to be pancreatic insufficient (311). They also had significantly more lung disease on chest CT as reflected by higher PRAGMA-CF scores (311). A further study which evaluated forskolin induced swelling (FIS) in organoids in patients with CF demonstrated a

correlation between FIS and intestinal current measurement (ICM) and sweat chloride (312). Addition of VX-770 to organoids with class III-V mutations resulted in an increase in organoid FIS. However, addition of VX-770 to organoids with class I-II mutations resulted in only a small increase in organoid swelling (312). The combination of VX-770 and VX-809 to organoids bearing at least one copy of the F508del mutation resulted in a synergistic increase in organoid swelling. Importantly a significant correlation was observed between organoids swelling and the absolute change in FEV_1 % predicted experienced by the patient (312). Two patients with CF with rare mutations – G1249R/F508del – were subsequently treated with ivacaftor on the basis of organoid swelling in response to VX-770 suggesting that they should derive a clinical benefit (312). Both patients experienced an improvement in nasal potential difference, sweat chloride, BMI and CFQ-R. One of the patients experience a 13% improvement in FEV_1, however, the second patient did not. Equally a patient with the F508del/R347P mutation which was not predicted to respond to ivacaftor based on organoid swelling data experienced no improvement with ivacaftor treatment (312).

Methods

Clinical data on this cohort of patients was already available as part of the ongoing CORK study (313). Ethical approval was obtained from the clinical research ethicscommittee of the Cork Teaching Hospitals for rectal biopsy sampling for organoidculture. A subset of patients in the CORK study consented to participate in this study. Change in clinical parameters was assessed using a paired Wilcoxon signed rank test.

Collection and transport of rectal biopsy

EQUIPMENT AND MATERIALS

Storage medium will be prepared at the site and stored for up to 2 months at 4ºC.

- 500ml Advanced DMEM/F12 [Invitrogen #12634-034]
- 5ml GlutaMax 100 x [Invitrogen # 35050-068]
- 5ml Hepes 1M [Invitrogen # 15630-056]
- 5ml Penicillin/Streptomycin 10K U/ml 10K µg/ml 100x [Invitrogen #15140-122]
- 500µl Primocin [Invivogen # ant-pm-1]
- 50 ml polypropylene test tube

Rectal biopsy samples are obtained in keeping with standard practice and transferred to a sample tube containing sample medium. Sample tubes are labelled immediately and stored at 4ºC. Samples are transferred at 4ºC in a sealed biologically secure transfer bag.

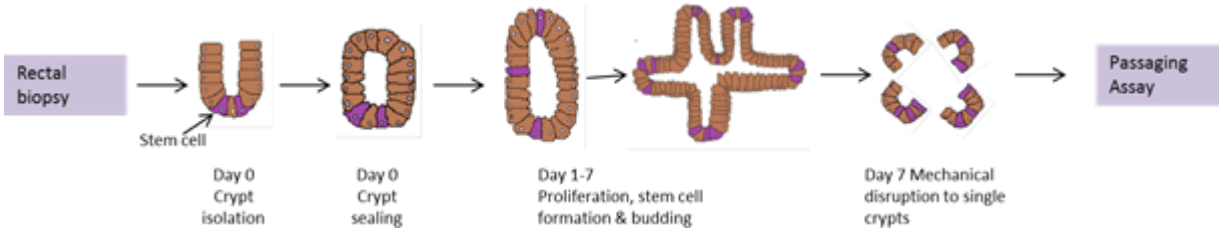


Figure 6.7 Rectal organoid isolation procedure

Crypt isolation and CF organoid culture establishment

EQUIPMENT

Biosafety cabinet, microscopy, pipette-aid, gas burner, rocking tube platform

MATERIALS

15ml falcon tubes (polypropylene, sterile), 10ml plastic pipettes, 70% ethanol, 200µl filter tips, P200, 24 well Cell Culture plates.

REAGENTS

- Ad-DF+++
- CF colon medium
- Isolation medium
- Matrigel, Basement Membrane Matrix, Growth Factor Reduced, Phenol Red, free (MG) (BD #356231), stored at -20ºC

  Note: once matrigel is thawed it must be transferred to a previously cold at - 20ºC 15ml tub using a -20ºC cold 5ml pipette.

  Note: If matrigel is already thawed and stored at 4ºC, it should be in ice at least 1 hour before starting using it. Make sure that MG is well resuspended (MG can polymerase if it's not cold). MG bottle takes about an over-night to thaw. Every new lot of MG should be tested to see if the percentage should be changed or kept as it is. MG must be thawed on ice (not on a fridge at 4ºC).
- EDTA (0.5M)
- PBSO

PROCEDURE

Always pre-wet your pipettes with Ad-DF+++. This will prevent the sticking of biopsies and crypts to the tube.

1. Remove the Ad-DF+++ from the 50ml falcon tube in which the biopsies are transferred.
2. Add 10ml of PBSO to the biopsies and pipette up and down 10-20 times using 10 ml pipette.
3. Let the biopsies settle at the bottom and remove supernatant.
4. Repeat steps 2 and 3 4-5 times until the supernatant is clear.
5. Add 10ml of PBSO to the biopsies.
6. Add 200µl EDTA (0.5 M) to 10 ml PBSO containing biopsies and place tube on rocking tube platform for 90-120 minutes at 4ºC. Incubation time may differ per patient. If crypts come loose, PBS seems cloudy. If crypts did not come out, add new PBSO + EDTA and incubate longer.
7. Allow biopsies to settle. Discard PBSO. Note: if crypts are floating (happens rarely) transfer supernatant to a new 15ml tube.
8. Prepare another tube with 5-15ml cold PBSO.
9. Using the pre-wet pipette, take up 2ml Ad-DF+++ land add it to a new 15ml tube. Shake this new tube manually in such a way that the inside is coveredwith Ad-DF+++.

10. Add 2-5ml PBSO from the PBSO tube (step 8) to the tube containing the biopsies, pipette up and down 10-20 times. Note: the crypts will loosen from biopsies and float in the PBSO, which is visible.
11. Allow biopsies to settle and transfer the PBSO with the crypts to the new tube containing the 2ml Ad-DF+++ which was prepared in step 9.
12. Repeat steps 10 and 11 until no more crypts get loose or until the content (PBSO + crypts +2ml Ad-DF+++) of the new tube reaches 12ml.
13. Spin crypts at 800 rpm (129g) for 5 minutes at 4ºC. In the meantime, transfer IM from ice to the safety cabinet where it comes to room temperature.
14. Discard supernatant and add 10ml Ad-DF+++ to the crypt pellet and repeat step 13.
15. After the centrifugation, the crypts will be in the pellet. Remove thesupernatant and resuspend the pellet in 55% MG. Resuspend crypts in enough volume of MG and seed 30µl per well (in a 24 well plate).
16. Place and leave the plate upside down in the incubator at 37ºC for 20 minutes for MG to solidify.
17. Add 500µl of IM per well and keep in the incubator at 37ºC and 5% CO_2.
18. Add fresh complete CM every 2-3 days.

Freezing organoids

EQUIPMENT

Biosafety cabinet, pipette-aid, waste container, centrifuge, CoolCell alcohol-free freezing containers, Liquid nitrogen tank, gas burner

MATERIALS

Sterile plastic pipettes, 70% ethanol solution, 15ml tubes, P1000, cryogenic vials

REAGENTS

- Ad-DF+++ stored at 4ºC
- Tryple E Express
- Recovery Cell Culture Freezing Medium (Invitrogen, #12648-010) stored at - 20ºC

PROCEDURE

Days in advance:

1. Incubate 24 well plates overnight at 37ºC.
2. If there is no thawed matrigel transfer 1 bottle (10ml) of thawed matrigel (MG) from -20ºC onto ice in a small box. Transfer the box to 4ºC fridge and thaw MG on ice at 4ºC overnight.
3. Label a 15ml falcon tube with MG quantity, date and initials.
4. Transfer the 15ml falcon tube and a 10ml pipette tip to -20ºC.

Day of passaging:

1. Start-up biosafety cabinet clean the surface work area and clean the P1000 and P200 pipettes with 70% ethanol.
2. Remove Tryple E from fridge and leave it at room temperature for at least 30 minutes before use.
3. Wipe all tubes off with 70% ethanol and transfer culture plate containing organoids and the supplemented medium into a biosafety cabinet.
4. Place one 15ml falcon tube on ice.
5. If necessary, remove the 15ml falcon tube and 10ml pipette from -20ºC and place on ice. Transfer the MG to the frozen 15ml falcon tube using the frozen 10ml pipette tip and put it back on ice.
6. Prepare fresh CF-CM+ RhoKi.
7. In the case that medium contains debris or dead cells, carefully aspirate the medium from the wells using a P1000 or a vacuum sucker and add 1ml of medium in two wells. If wells are clean, remove the medium from all wells except four. The medium will be used to collect the organoids.
8. To disrupt organoids, pipette the medium in the well and break up the MG by pipetting up and down using a P1000 pipette.
9. Transfer the solution to the next well that needs to be disrupted. After disrupting all the wells, transfer the solution into the 15ml tube.
10. Fill the 15ml tube up to 12ml of Ad-DF+++. Pipette up and down using a pre-wet 10ml pipette.

11. Spin at 650 rpm (85g) for 5 minutes at 4ºC.
12. Remove as much supernatant as possible.
13. Add 5ml of Tryple-E and pipette up and down with a 5ml pipette several times.
14. Put the tube in a warm water bath at 37ºC for 1 minute and vortex vigorously for 30 seconds.
15. Check the solution in the tube. If organoids are still visible by eye, repeat step 14.
16. When organoids are not visible, add 7-10ml of cold Ad-DF+++ to neutralise the Tryple-E.
17. Centrifuge at 4ºC for 5 minutes at 1500rpm (453g).
18. Discard supernatant and add the required amount of medium and MG to the organoids to reach a 55% MG solution. Mix by pipetting up and down without creating bubbles. For freezing, organoids must be seeded in a 1:1 ratio after trypsinisation.
19. Seed 250µl to a single well of pre-warmed 6-well plate, making tiny, separated droplets of 10µl. place the plate upside down in the incubator at 37ºC and leave MG to solidify for 20-30 minutes.
20. Add 2.5ml fresh CM+RhoKi per well plate and transfer the plate back to the incubator. To avoid any excess of MG, 3 wells of a 6-well plate will be pooled in 1 tube of 15ml. if you are freezing a full 6-well plate you must use 2 15ml tubes.
21. Fill up the 15ml tubes containing the organoids until 12ml with cold Ad-DF+++. Pipette up and down with a 10ml pipette and put the tube in ice.

22. Pipette up and down again several times with a 10ml pipette or vortex.
23. Centrifuge the organoids for 5 minutes at 1500 rpm (453g).
24. Take off the supernatant. In the case of accidental removal of the pellet, re-suspend and spin down again.
25. If after centrifugation there is more than 1ml of MG in each 15ml tube, repeat steps 21-24.
26. Dissolve the pellet of each 15ml tube with 2.5ml of Recovery Cell freezing medium and transfer them to a 50ml falcon tube.
27. Add the necessary volume of Recovery Cell freezing medium and pipette up and down to re-suspend the organoids. 1000µl of freezing medium will be used for each 100µl of MG.
28. Using a cold 5ml pipette, transfer 1000µl of organoids re-suspended in freezing medium should be transferred to sterile cryogenic vials.
29. Transfer the vials to a Mr Frosty and put at -80ºC.
30. After 24 hours at -80ºC transfer the vial to the liquid nitrogen tank.

Starting cultures from frozen organoids

REAGENTS

- Ad-DF+++ stored at 4ºC
- PBSO
- CF-CM (CF- colon medium)
- Matrigel
- Rho kinase inhibitor (RhoKi)
- 1 vial of frozen organoids

PROCEDURE

Days in advance

1. Put a 24-well tissue culture plate overnight in the incubator to warm up to 37ºC.
2. Check that you have thawed matrigel. If no transfer 1 bottle (10ml) from -20ºC onto ice in a small box and let it thaw overnight inside of refrigerator (4ºC).
3. Label a 15ml falcon tube with MG, date and initials.
4. Transfer the 15ml falcon tube and a 10ml pipette tip to -20ºC.

Day of procedure

1. Ad-DF+++ medium should be out of the fridge at least 30 minutes to reach room temperature and warm up 10ml at 37ºC before starting the procedure. The thawed cell solution should be dilute on warm Ad-DF medium as quickly as

possible. If cells remain in the freezing medium for too long the viability will be poor.

2. Thaw the vial rapidly by agitation in at 37ºC water bath until there is still little frozen material (this will thaw further form the moment you take the vial out of the water bath until the moment you proceed). Thawing should be rapid (within 60-120 seconds). Remove the vial from water bath and wipe it down with 70% ethanol and transfer to a biosafety cabinet.
3. In the biosafety cabinet, transfer the thawed organoids to a sterile 15ml falcon tube using a P1000 pipette. Immediately after, add 1ml of warm Ad-DF+++ drop by drop while shaking the bottom of the tube. Once the 1ml of medium is added, mix carefully by pipetting up and down a few times to dilute the freezingmedium.
4. Slowly (drop by drop) add 9ml of warm Ad-DF+++ into the falcon tube containing the organoids, using a 10ml pipette. Invert a few times.
5. Centrifuge the cell suspension at 4ºC, 650 rpm (85g) for 3 minutes. Higher speeds will affect viability of the cells negatively because the membranes are more sensitive due to the thawing process.
6. Discard the supernatant carefully without disrupting the pellet.
7. Re-suspend the pellet in 90µl of CF-CM including RhoKi (1:1000 dilution from stock). Then add 110µl of matrigel so the organoids are seeded in double volume of matrigel that was frozen in the vial (100µl). Matrigel and pelleted organoids should stay on ice during this procedure.

8. Add 35-40µl to each well of pre-warmed 24-well plate, making tiny separated droplets. Then transfer the plate to the 37ºC incubator leaving the plate upside down for 20-30 minutes for matrigel to solidify.
9. Add 500µl of CF-CM with RhoKi to each well. Matrigel drops should be very crowed. This helps organoids to recover from thawing process.
10. Transfer the plate back to the incubator. Re-fresh the medium three times per week.

Passaging of intestinal organoids

EQUIPMENT

Biosafety cabinet, pipette-aid, waste container, centrifuge, incubator (37ºC, 5% CO_2), vortex mixer, gas burner, water bath

MATERIALS

Sterile polystyrene pipettes 5ml and 10ml (Greiner), Pasteur pipettes, Low retention filter tips 1250, 300 and 20 µl, Easyload 200 and 10 µl plastic tips, 6, 12 and 24-well Cell culture plates

REAGENTS

- Stock medium (Ad-DF+++), stored at 2-8ºC
- Normal intestine/colon medium
- Matrigel (MG)

Days in advance:

1. Pre-warm 24 well plates at least overnight in the incubator at 37ºC.
2. Check that you have thawed Matrigel. If not transfer 1 bottle (10ml) from -20ºC onto ice in a small box and let it thaw overnight inside a refrigerator at 4ºC.
3. Label a 15ml falcon tube with MG, date and initials.
4. Transfer the 15ml falcon tube and a 10ml pipette tip to -20ºC.

Day of passaging

5. Start-up biosafety cabinet clean the work surface and clean the P1000 and P200 pipettes with 70% ethanol.
6. Wipe all tubes/ containers off with 70% ethanol and transfer culture plate containing organoids and supplemented medium into a biosafety cabinet.
7. Place two 15ml tubes in the biosafety cabinet. Label one of the tubes as washes. Disrupted organoids will be collected in this tube.
8. Remove the 15ml falcon tube and 10ml pipette from -20ºC and place them on ice.
9. Transfer the 10ml of Matrigel to the frozen 15ml falcon tube using the frozen 10ml pipette tip in the biosafety cabinet and put the tube back on ice.
10. Also have CF-CM + RhoKi ready. RhoKi stock [10mM] is 1000x concentrated, so transfer the volume of CF-CM needed for the passaged sample into a falcon and add RhoKi (e.g. For 5ml of CF-CM add 5µl of RhoKi).

11. In case that medium contains debris od dead cells, carefully aspirate the medium from the wells using a P1000 or a vacuum sucker and add 1ml of medium in one well. If wells are clean, remove the medium from all wells except two. The medium will be used to collect the organoids.

12. To disrupt the organoids, pipette the medium in the well and break up the MG by pipetting up and down using a P1000 pipette.

13. Transfer the solution to next well that needs to be disrupted. After disrupting all the wells, transfer the solution into the 15ml pipette.

14. Add a white (for P2 or P10) or a yellow (for P20 or P200) tip on top of the 1ml pipette tip. Shear the organoids by pipetting it at a 45º angle with the tip against the side-bottom of the 15ml tube. Pipette up and down 20 times making sure that the fluid goes completely through the tips every time.

15. Add 4ml of Ad-DF+++ to the sheared organoids.
16. Remove the small (white or yellow) tip from the 1ml tip for the collection step.
17. Hold the tube at a 45º to 80º angle and pipette the 5ml solution up and down to re-suspend the organoids.
18. Count 10 seconds and collect the organoids by putting the tip at the top of the liquid and carefully collect 4ml of the disrupted organoids. Observe how undisrupted/ differentiated organoids and bigger chunks will sink to the bottom.
19. Spin the collected 4ml at 800rpm (129g) for 2 minutes at 4ºC.

20. Discard supernatant and add 55-45% Matrigel/CF-CM solution to the cell pellet for plating.
21. Seed organoids with a P200 by pipetting around 35-40µl per well (from 3 to 5 drops per well of a 24 well plate).
22. Place the plate upside down in the incubator at 37ºC and leave Matrigel to solidify for 20-30 minutes.
23. Add 500µl of fresh CF-CM+RhoKi per well and transfer the plate back to the incubator.
24. After 7-10 days, split organoids following the same procedure. The usual split ratio for expanding CF organoids is 1:3.

Plating organoids

Not all organoid cultures grow nicely (fast and without showing cell death or signs of differentiation) in 6 well plates. If the growth is not what is expected, culture the organoids in 24-well plates. Organoid density is an important parameter for the correct growth of organoids.

1. After the centrifugation, check under the microscope to see the border between the gel and the pellet.
2. Aspirate the supernatant and get as close as possible to the pellet. Do not touch the pellet.
    a. Estimate the volume of the sheared organoids in medium left. The pellet + medium need to be resuspended in a mixture of 30% of the

supplemented organoid culture medium and 70% MG (in this order). Refer to the calculation table below for different dilutions.

Table 1: Per well of a 6-well plate, 200µl of MG containing organoids are seeded. Below are calculations of the different dilutions for plating different number of wells.

Table 6.2 Dilution

# of wells	Organoid medium (µl)	MG (µl)	Total (µl)
1	60	140	200
2	120	280	400
3	180	420	600
4	240	560	800
5	300	700	1000
6	360	840	1200

Note: Seed the total volume of MG containing organoids in several drops of 10µl maximum. Check the dilution always with 1 drop and if needed dilute further.

b. In cases of budding organoids (e.g. Cystic fibrosis) resuspend the pellet in 55%MG/45% normal colon organoid medium. Usually CF organoids are split 1:2 or 1:4 and seeded in 35µl MG/CF organoids per well in a 24-well plate. Follow the table to dilute the organoid pellet. Note: Seed 15- 20 organoids per 5µl of 55% MG. To check it, plate 2-3 drops of 5µl and check the number of organoids under the microscope. Dilute it further if needed.

Table 6.3 Matrigel dilution			
24 well plate (55%)			
Wells	Media (µL)	Matrigel (µL)	Total (µL)
1	15.75	19.25	35
2	31.5	38.5	70
3	47.25	57.75	105
4	63	77	140
5	78.75	96.25	175
6	94.5	115.5	210
7	110.25	134.75	245
8	126	154	280
9	141.75	173.25	315
10	157.5	192.5	350
11	173.25	211.75	385
12	189	231	420
13	204.75	250.25	455
14	220.5	269.5	490
15	236.25	288.75	525
16	252	308	560
17	267.75	327.25	595
18	283.5	346.5	630
19	299.25	365.75	665
20	315	385	700
21	330.75	404.25	735
22	346.5	423.5	770
23	362.25	442.75	805
24	378	462	840

3. Seed 200 µl per well (6-well plate), 100 µl per well (12-well plate), 50 µl per well (24-well plate) in small drops, to improve the diffusion of growth factors into the MG. avoid creating air bubbles while seeding.
4. After seeding, turn the seeded plate upside down and leave it in the hood for 5-10 minutes. This will prevent the bigger organoids from sinking to the bottom of the well.

5. Incubate the plate upside down at the corresponding incubator for 20-30 minutes to solidify the MG drops.
6. Transfer the plate back to the hood.
7. Print plate label and stick on the side of the plate.
8. Add 2 ml (6-well plate), 1ml (12-well plate), 500 µl (24-well plate) of organoid culture medium with RhoKI (10µM final concentration) per well and place the plate in the incubator at 37ºC and 5% CO_2.
9. The organoids are refreshed with organoid culture medium every 2-3 days.
10. Organoids are usually passaged every 7-10 days depending on their growth and density.

Forskolin induced swelling (FIS) Organoid assay Day 1

EQUIPMENT

Biosafety cabinet, Yokogawa microscope, pipette-aid, pipette-set (P1000, P200, P20), centrifuge, incubator

MATERIALS

Multichannel pipette, Viaflo pipette, Viaflo tips, P200 multichannel pipette tips, 1.5ml Eppendorf tubes, 15ml falcon tubes, 5ml & 10ml plastic pipettes, 70% ethanol, sterile 96 well-plate (flat bottom)

REAGENTS

Forskolin (Tocris #1099, stock 10mM, stored at -80ºC), Ad-DF+++, DMSO aliquoted and stored at -20ºC, CF colon medium (CM), VX-809 (Selleckchem #S1565) stock aliquots at 20mM stored at -80ºC, VX-770 (Selleckchem #S1144) stock aliquots at 20mM stored at -80ºC, Calcein (Molecular probes #C3100MP) stored at -20ºC, Matrigel (Corning) stocks stores at -80ºC

PROCEDURE

In this assay, CFTR function is measured. Organoids are treated with VX-809, VX-770 and in combination for CF biobank characterisation purposes. Forskolin is used at four different concentrations, while VX-809 and VX-770 are used at 3µM.

Day of plating

1. Prepare the required amount of CF CM
2. Remove an aliquot of VX-809 (20mM) from the -80ºC freezer and protect it from light. Note: any drug re-suspended in DMSO must be thawed at room temperature at least 1 hour before being used in assay.
3. Process organoids as described in LM 5 (mechanical sheering) until step xi.

   Note: depending on the amount and size of the organoids between 4 and 6 wells of a 24-well plate should be used for seeding one assay for one organoid culture (48 wells of a 96 well plate).
4. Resuspend the pellet in 50% Matrigel (MG). Note: usually 210 µl of 50% MG is used to resuspend an organoid pellet processed from 4-6 wells.
5. Confirm the number of organoids (30-50) in a 3 µl MG drop under the microscope. If there are more organoids, dilute the sample with more 50% MG. if there are less organoids, start from step 3 of this lab manual and resuspend the new pellet of organoids with the previous one already in 50% MG.
6. Plate organoids using a single drop of 3µl. Note: Total number of wells for one organoid culture is 48 and the amount of MG needed is 214 µl including the 10% error rate.

   Note: representative drawing of the plate layout for organoid culture characterisation. In a 96-well plate, two samples can be plated. Indicate new layout in case it is different in the template FIS assay form.
7. Place the plate in the 37ºC incubator for 15 minutes for MG to solidify.

8. In the meanwhile, prepare VX-809 solution for at least 5ml of CM
    a. Add the 5µl aliquot of VX-809 (20mM) into 45µl DMSO (final concentration 2mM)
    b. Add 7.5µl VX-809 [2mM] to 5ml of CF CM full medium (final concentration 3µM) (label the tube as CF CM + VX-809)
9. Using the Viaflo pipette, add 100µl of regular CF CM to each well without VX- 809 (labelled as DMSO and VX-770).
10. Add 100µl of CM + VX-809 (prepared at step 8) to each well that must be incubated with VX-809. Note: VX-809 solutions and aliquots should not be reused and discarded after use. VX-770 will be added to wells labelled with VX- 770 on the day of running the assay.

Day 2 Day of measuring

Note: make sure VX-809 is incubated about 16-20 hours before starting this procedure. The calculations are based on one sample (48 wells). In case of running 2 samples on a plate, use double the volume.

1. Before making the drug solutions, turn on the Yokogawa microscopy.
2. Take a vial of calcein and DMSO and leave them at room temperature for 15 minutes, if needed.
3. Number and label the tubes that will be used for Forskolin and/or VX-770 solutions.
4. Add 5.2µl of DMSO to a calcein vial, if unopened. Otherwise, use an already resuspended calcein vial containing 2.5µl of calcein in DMSO. Note: calcein is

sensitive to light therefore make sure that the light in the hood is turned off or keep in a dark area.

5. Add 2.5µl calcein in DMSO to 580µl Ad-DF+++ in an Eppendorf and label the tube calcein.
6. Add 10µl of this dye solution (Ad-DF+ calcein) to each well using a Viaflo repeat pipette.
7. Resuspend once with a multichannel to ensure the dye and CM are mixed well. Note: to prevent making bubble or resuspending MG when mixing, set the pipette to 50µl.
8. Incubate the plate in a 37ºC incubator for 30 minutes. Note: the dye must be added 60 minutes before starting the measurement. Beware that the marking position of each well and adding the solution will also take time which also needs to be considered.
9. During calcein incubation, start preparing Forskolin and VX-770 solutions.
    a. Add 15µl Forskolin [10mM] to 15ml Ad-DF+++ (= Forskolin [10µM], final concentration in assay will be 5µM). Note the final concentration in the assay will be 5µM as 100 µl of titration solution will be added to each well which already contains 100µl of CM, thereby lowering the Forskolin concentration by half.
    b. Add 4.8µl of VX-770 stock [20mM] to 15 ml Ad-DF+++ (=VX-770 [6.4µM], final concentration in assay will be 3.2µM).

c. Take 5ml VX-770 (6.4µM) and add 5 µl Forskolin [10mM] (=Forskolin [10µM] +VX-770 [6.4µM]. Note: all calculations and numbering of the tubes must be done in advance in order to prevent any delay.

d. For basal Forskolin response and VX-809 treatment (DMSO and VX-809 wells) prepare concentrations as described below. Resuspend allsolutions 10 times before collection solution for next tube.

Forskolin final concentration (µM)	Preparation
10	Is already prepared (10µM)
1.6	160µl from 10µM solution in 840µl Ad-DF+++
0.256	160µl from 1.6µM solution in 840µl Ad-DF++
0.04	160µl from 0.256µM solution in 840µl Ad-DF++

e. For VX-770 treatment (VX-770 and VX-770/VX-809 wells) prepare concentrations as described below. Resuspend all solutions 10 times before adding solution to the next tube.

Forskolin + VX-770 (µM)	Preparation
10	Is already prepared
1.6	160µl from 10µM solution in 840µl VX-770 6.4(µM)
0.256	160µl from 1.6µM solution in 840µl VX-770 6.4(µM)
0.04	160µl from 0.256µM solution in 840µl VX-770 6.4(µM)

10. After the calcein incubation of the organoids, aspirate the solution from each well carefully.

11. Add 100µl of CF CM medium to each well very carefully, avoiding the disruption of the Matrigel drop using repeat pipette.
12. Transfer the Forskolin and VX-770 solutions (described in step 9) using a P200 pipette and tips to the Yokogawa microscope.

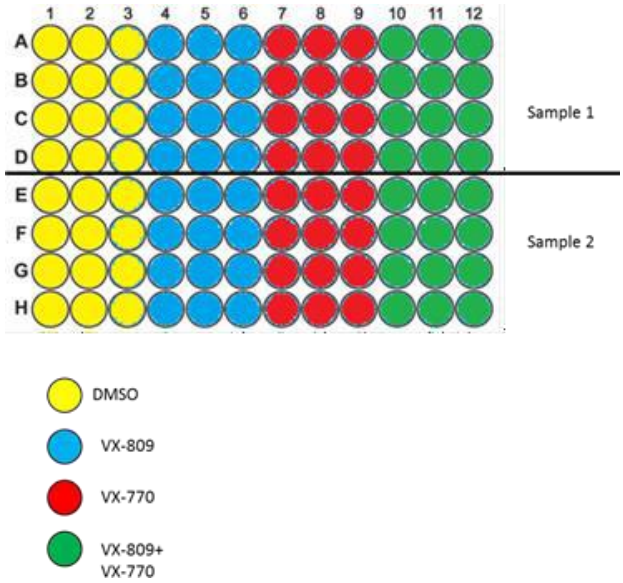


Figure 6.8 Plate layout with CFTR modulators

13. Place the 96 well plate on the stage and configure acquisition points.
14. Add 100µl of the Forskolin or Forskolin +VX-770 titrations into the corresponding wells as per the figure below.

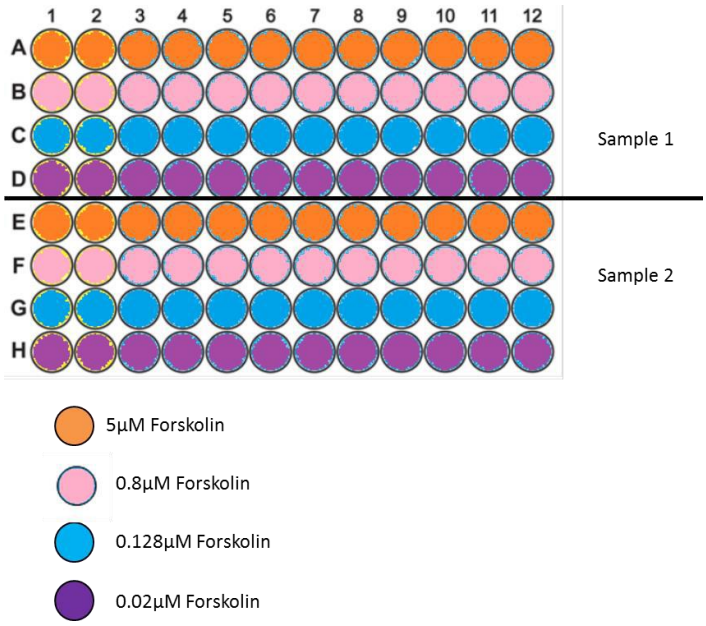


Figure 6.9 Plate layout with forskolin

Statistical Analysis

Data was not normally distributed, thus paired sample Wilcoxon signed rank test was used to assess change in clinical parameters before and after ivacaftor. Spearman rank correlation was used to evaluate for relationship between FIS and change in clinical parameters after treatment.

Results

Ten adult patients with CF with the G551D mutation treated with ivacaftor consented to rectal biopsy for subsequent culture of rectal organoids. Figure 6.10 demonstrates the maximum forskolin induced swelling in the absence of ivacaftor. Based on these results Cork CF 008 and Cork CF 010 were excluded from further analysis as the level of

baseline organoid swelling in the absence of ivacaftor was above the expected threshold.

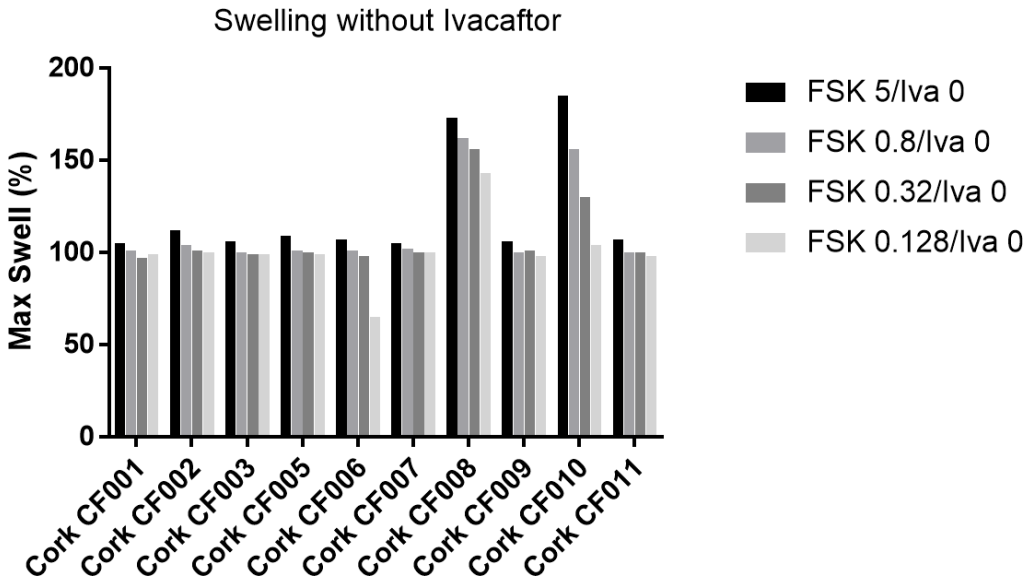


Figure 6.10. Forskolin induced organoid swelling in the absence of ivacaftor (maximum swelling measures in the presence of different concentrations of forskolin)

These patients carried the genotypes G551D/unknown and G551D/R117H respectively. The patient with the genotype G551D/unknown was diagnosed with CF based on recurrent chest infections, chest CT showing bilateral upper lobe predominant bronchiectasis, osteoporosis and sputum growing *Staphylococcus aureus*. Table 6.4 summarises patient's clinical response to ivacaftor. Both patients experienced improvements in FEV_1 and BMI post ivacaftor.

Table 6.4 Change in clinical parameters for two excluded patients						
Patient number	Genotype	Age	Baseline FEV_1	Mean Δ FEV_1	Mean Δ Sweat	Mean ΔBMI
CF008	G551D/unknown	52	48%	10%	+11mmol/l	1kg/m^2
CF010	G551D/R117H	44	48%	4%	-46 mmol/l	2.7kg/m^2

The cohort included in the final analysis thus comprised 8 patients. Table 6.5 summarises the baseline clinical characteristics of these patients.

Table 6.5 Baseline characteristics before ivacaftor (n=8)	
Characteristic	**Median (Range)**
Age (years)	32.5 (25-42)
Gender (% male)	50%
Genotype	
F508del/G551D	75%
F508del/3028delA	12.5%
G551D/G551D	12.5%
FEV_1 (%predicted)	49 (30-91)
Sweat chloride (mmol/l)	107.5 (64-155)
BMI	23 (18.2-29.5)
CFQR Respiratory domain	66.7 (22.2-100)
Median IV antibiotics	0.5 (0-5)

Statistically significant improvements in FEV_1 % predicted, sweat chloride, and CFQ-R respiratory domain were observed after ivacaftor (Table 6.6). A non-statistically significant improvement in BMI was observed after 1 year of ivacaftor. Significant improvement in chest CT total Bhalla score was observed after 1 year of treatment. A 70% reduction in IV antibiotic usage was observed in the year after ivacaftor. Table 6.6 summarises clinical change after ivacaftor.

Table 6.6 Median change in clinical parameters after ivacaftor		
Parameter	Median change (range)	*P value*
FEV_1 % predicted	10.25 (-3.5 – 16.5)	*0.025*
Sweat chloride	-66 (-24 to – 107)	*0.018*
BMI	0.75 (-2 – 1.7)	*0.123*
CFQ-R respiratory domain	16.6 (-10.9 – 44.5)	*0.026*
Bhalla score	-2 (-1 to -3)	*0.026*

A non-statistically significant positive correlation was observed between organoid swelling after treatment with ivacaftor (Ivacaftor 1 μM /Forskolin 0.128 μM) and the change in FEV_1% predicted at 6 (r =0.59, *p=0.17*), 9 (r =0.69, *p=0.058*), 12 months (r =0.61, *p=0.1*) and the average FEV_1 % predicted in the year after ivacaftor (r = 0.64, *p> 0.05*) using a Spearman rank correlation test (Figure 6.11) .

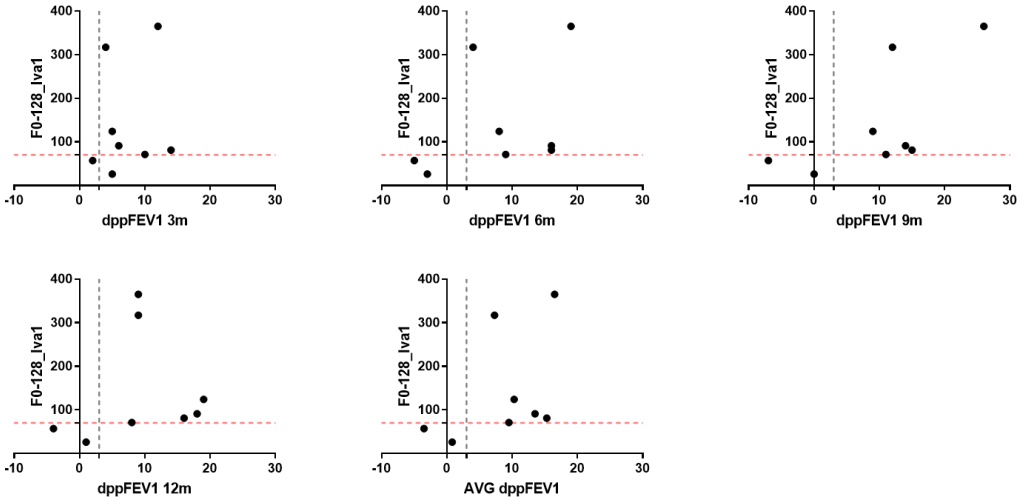


Figure 6.11. Scatter plot demonstrating change in FEV_1 % predicted and organoidswelling (Grey dotted line indicates change in FEV_1 ≥3%. Red dotted line indicates cut- off between organoid responders and non-responders).

There was no statistically significant correlation between change in sweat chloride and organoid swelling at 3 (r = 0.69, *p= 0.058*) or 12 months (r = 0.57*, p = 0.18*). There was no significant correlation between change in chest CT total Bhalla score and organoid swelling at 3 months (r = 0, *p = 1*) or 12 months (r = -0.27, *p = 0.57*). There was no significant correlation between change in BMI and organoid swelling at 3 months (r = 0.13, *p = 0.75*), 6 months (r=0.48, *p =0.23*), 9 (r = 0.36, *p =0.4*). The correlation between change in BMI and organoid swelling was statistically significant at 12 months (r = 0.73, *p = 0.038*). There was no significant correlation between change in CFQ-R resp domain and organoid swelling at 3 (r = 0.2, *p = 0.7*) or 12 (r=0.45, *p =0.3*) months.

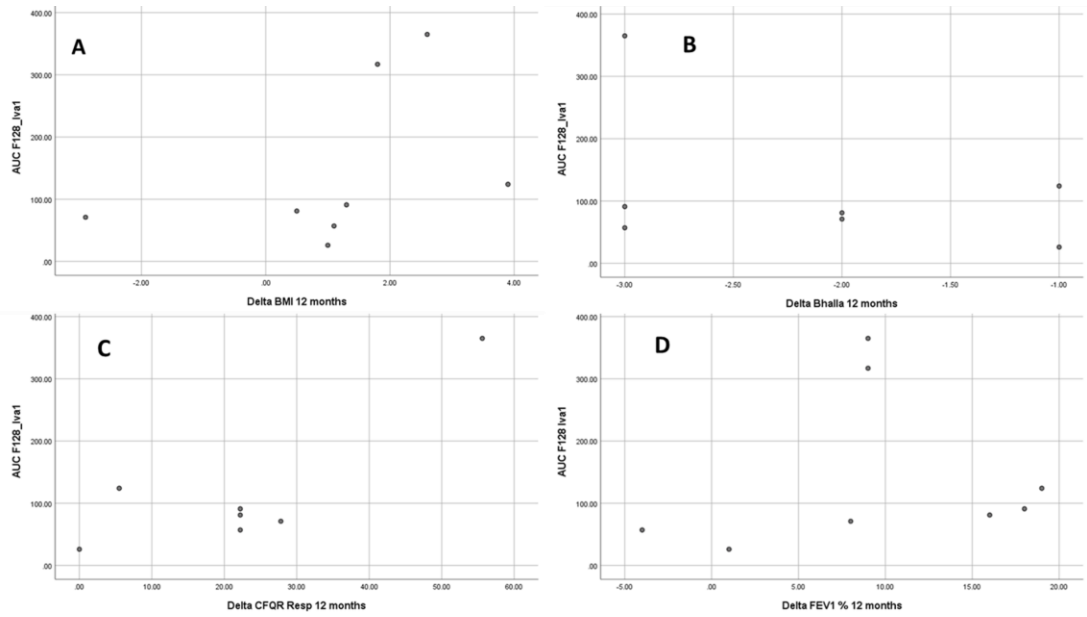


Figure 6.12 no significant correlation between FIS and change in clinical parameters after 12 months of ivacaftor.

One patient in the cohort (CF006) was homozygous for the G551D mutation. He has a baseline FEV_1 of 91% predicted pre-treatment. He experienced a 16% increase in FEV_1, a -77mmol/l reduction in sweat chloride, 0.5kg/m^2 increased in BMI and 2-point improvement in chest CT Bhalla score after 12 months of ivacaftor. This patient'sbaseline organoid swelling after forskolin but without ivacaftor was at the median for the cohort. After addition of ivacaftor organoid swelling in this patient was below the median despite carrying two copies of the G551D mutation and experiencing the largest increased in FEV_1 of the cohort.

Discussion

Two patients had baseline organoid swelling greater than expected and thus were excluded from final analysis. However, despite this higher background organoid swelling both had significant respiratory impairment with both having a baseline $FEV_1$of 48% predicted. Additionally, both experienced clinically meaningful improvements in FEV_1 and BMI after ivacaftor treatment. Thus, in milder mutations this may not be the optimal method of predicting treatment response.

In a clinically responsive cohort, we did not demonstrate a statistically significant correlation between forskolin induced organoid swelling and change in FEV_1% predicted at 3, 6, 9 and 12 months after treatment. This is in contrast with previous work demonstrating a correlation between FIS and delta FEV_1 % predicted in patients with CF with a variety of residual function mutations (312). We did not demonstrate a significant correlation between other clinical parameters including BMI, CFQ-Rrespiratory domain, sweat chloride, BMI and chest CT Bhalla score. One of the

limitations of this study is the small sample size of 8 participants included in the final analysis and thus this may have limited our ability to detect a significant correlation.

Conclusion

This chapter highlights the concept of precision medicine and assessing the benefit of a therapy in each patient as an individual across multiple parameters. This may be particularly relevant in an orphan condition like CF in which there may be very small numbers of patients with some of the rarer mutations therefore, making large clinical trials impossible.

Chapter 7

Conclusion

Large multi-centre phase II and III clinical trials are a pre-requisite to final approval of medications by regulatory bodies. However, there is a significant cost associated with them. Consequently, it is necessary to have reliable outcome measures which can accurately and reliably forecast treatment response. Additionally, clinical trials have strict enrolment criteria and the patients included may not be representative of the patients who ultimately receive the medication in a real-world setting. This thesis evaluated the effect of ivacaftor on a cohort of real-world patients with CF and demonstrated effectiveness across a variety of clinical parameters including FEV_1, pulmonary exacerbations, CFQ-R, and modified shuttle walk test.

Traditionally in CF the most common primary outcome measures are FEV_1 and pulmonary exacerbation frequency. However, as described in chapter 2 these outcome measures not without their limitations. FEV_1 is subject dependant, cannot be performed by very young patients and can be relatively insensitive to structural decline. Equally as larger numbers of people with CF reach adulthood with relatively preserved lung function larger numbers of patients are required to participate in studies to demonstrate a significant FEV_1 improvement. Similarly, there are limitations associated with using pulmonary exacerbation frequency. There is no standard definition of what constitutes a pulmonary exacerbation and as care improves exacerbations may be a relatively infrequent occurrence thus requiring larger numbers of patients to participate in studies. In chapter 2 we demonstrated a significant improvement in low dose chest CT after treatment. This raises the potential utility of low dose chest CT in CF studies. Unlike FEV_1 it is not effort dependent, can be

performed at any age and chest CT changes have been observed in asymptomatic infants with CF as young as 6 months of age. Thus, this may be a useful outcome measure in patients with relatively preserved lung function. Similarly, it would beimportant to assess the ability of CFTR modulatory therapy to prevent or delay the progression of bronchiectasis on low dose chest CT as this would support early initiation of therapy. We demonstrated an increase in sputum microbial diversity and richness and a reduction in the relative abundance of *Pseudomonas aeruginosa* after treatment. Reduced sputum diversity and richness and acquisition of *Pseudomonas aeruginosa* are associated with worse respiratory function. Thus, ivacaftor may modify the traditional course of CF pulmonary disease.

The global burden of disease study 2013 demonstrated an increase in the number of people living with multiple comorbidities, with the aging of the global population (197). 32% of adults aged 20-64 have 5 or more comorbidities and the number of comorbidities in an individual increased with age (197). Life expectancy is increasing with CF. A recent European CF Registry study highlighted that by the year 2025 there would be a 75% increase in adults with CF (198). These calculations were based on a cohort of patients who were CFTR modulatory therapy naive. CFTR modulation treatment offers a new personalised approach to CF care and given its systemic mode of action there is potential for modification of both pulmonary and non-pulmonary CFTR disease and outcome (199). Given the improved CF survival with traditional approaches, the potential of augmenting this by new CFTR modulation, whilst working in a clinical arena where morbidities in aging populations (independent of CF) are a

greater issue. Thus, it raises the question of whether the ability of a treatment to delay or prevent the development of CF associated comorbidities needs to be factored into how new therapies are evaluated. We have demonstrated significant improvements in circulating inflammatory markers, subtle changes in gut microbiota, but no significant changes in exocrine or endocrine pancreatic function or gut inflammation. The excess inflammation and changes in gut microbiota observed in CF may be multi-factorial in aetiology and not solely due to CFTR dysfunction. Whether a longer period of CFTR modulation is required to reverse some of these abnormalities remain to be fully elucidated. The mixed effects of CFTR modulatory therapy on CF comorbidities suggests this requires longer term assessment.

In a paediatric cohort significant improvement in exocrine pancreatic function were observed with treatment, however, in an adult cohort we demonstrated no significant change in faecal elastase 1 after ivacaftor. This suggests that exocrine pancreatic function is irreversibly lost in an adult cohort and cannot be enhanced with CFTR modulatory therapy. This would support the early initiation of ivacaftor in a paediatric cohort as adult pancreatic insufficiency appears to be irreversible. CFRD is a common CF associated comorbidity with prevalence increasing with age.

CF is an autosomal recessive orphan condition. Over 1800 CF associated mutations have been identified. The benefit to an individual patient may be unique to that observed in the clinical trials. In patients with rarer mutations it may be difficult to conduct large randomised controlled trials due to limited patient numbers, thus n-of-1 and individual patient trials may have a role. In chapter 6 we demonstrate the utility of

ivacaftor to the individual patient in a series of unique cases. We report the utility of ivacaftor in reducing hospitalisation, pulmonary exacerbations and improving quality of life in a patient with severe R117H CF. We report similar improvements across a range of clinical parameters in two siblings with G551D/F508del CF, one receiving standard twice daily ivacaftor and the other receiving twice weekly treatment due toconcomitant Itraconazole usage. In this chapter we report on an older child with a partial restoration of exocrine pancreatic function after treatment and an adult whose main benefit from ivacaftor is improved gastrointestinal symptomatology. We demonstrated no significant correlation between forskolin induced organoid swelling and change in FEV_1% predicted, BMI, sweat chloride, CFQ-R respiratory domain or CT Bhalla score after ivacaftor. Additionally, two patients with milder mutations but significant respiratory impairment had baseline FIS which was above baseline threshold but still experienced clinical improvement after ivacaftor. Thus, there may be cohorts such as those with residual function mutations for whom FIS is not the optimal methodof predicting treatment response.

These are exciting times in cystic fibrosis with the recent FDA approval of trikafta (elexacaftor (VX445)/ivacaftor/tezacaftor) and the potential to treat 90% of patients with CF with modulator therapy. Given the potential of CFTR modulators to delay the progression of lung disease comorbidities and multidisciplinary management of increasingly complex patients will be important. It will also be enlightening to observe the long-term implications of early CFTR modulation on comorbidities.

Appendix 1: Cystic Fibrosis Questionnaire Revised (CFQ-R)

CFQ-UK

Adolescents and Adults (Patients 14 Years Old and Older)

CYSTIC FIBROSIS QUESTIONNAIRE - REVISED

Understanding the impact of your illness and treatments on your everyday life can help your healthcare team keep track of your health and adjust your treatments. For this reason, this questionnaire was specifically developed for people who have cystic fibrosis. Thank you for your willingness to complete this form.

Instructions: The following questions are about the current state of your health, as you perceive it. This information will allow us to better understand how you feel in your everyday life.

Please answer all the questions. There are **no** right or wrong answers! If you are not sure how to answer, choose the response that seems closest to your situation.

Section I. Demographics

Please fill-in the information or tick the box indicating your answer.

A. What is your date of birth?

Date ☐☐ ☐☐ ☐☐☐☐
Day Month Year

B. What is your gender?

☐ Male ☐ Female

C. During the **past two weeks**, have you been on holiday or out of school or work for reasons **NOT** related to your health?

☐ Yes ☐ No

D. What is your current marital status?

☐ Single/never married
☐ Married
☐ Widowed
☐ Divorced
☐ Separated
☐ Remarried
☐ With a partner

E. Which of the following best describes your racial background?

☐ White - UK
☐ White - other
☐ Indian/ Pakistani
☐ Chinese/ Asian
☐ African
☐ Caribbean
☐ Other [not represented above or people whose predominant origin cannot be determined/ mixed race]
☐ Prefer not to answer this question

F. What is the highest level of education you have completed?

☐ Some secondary school or less
☐ GCSEs/ O-levels
☐ A/AS-levels
☐ Other higher education
☐ University degree
☐ Professional qualification or post-graduate study

G. Which of the following best describes your current work or school status?

☐ Attending school outside the home
☐ Taking educational courses at home
☐ Seeking work
☐ Working full or part time (either outside the home or at a home-based business)
☐ Full time homemaker
☐ Not attending school or working due to my health
☐ Not working for other reasons

CFQ-UK

Adolescents and Adults (Patients 14 Years Old and Older)

CYSTIC FIBROSIS QUESTIONNAIRE - REVISED

Section II. Quality of Life

Please tick the box indicating your answer.

*During the past **two weeks**, to what extent have you had difficulty:*	A lot of difficulty	Some difficulty	A little difficulty	No difficulty
1. Performing vigorous activities such as running or playing sports	☐	☐	☐	☐
2. Walking as fast as others	☐	☐	☐	☐
3. Carrying or lifting heavy things such as books, shopping, or school bags	☐	☐	☐	☐
4. Climbing one flight of stairs	☐	☐	☐	☐
5. Climbing stairs as fast as others	☐	☐	☐	☐

***During the past two weeks**, indicate how often:*	Always	Often	Sometimes	Never
6. You felt well	☐	☐	☐	☐
7. You felt worried	☐	☐	☐	☐
8. You felt useless	☐	☐	☐	☐
9. You felt tired	☐	☐	☐	☐
10. You felt full of energy	☐	☐	☐	☐
11. You felt exhausted	☐	☐	☐	☐
12. You felt sad	☐	☐	☐	☐

Please circle the number indicating your answer. Please choose only one answer for each question.

*Thinking about the state of your health over the last **two weeks**:*

13. To what extent do you have difficulty walking?
1. You can walk a long time without getting tired
2. You can walk a long time but you get tired
3. You cannot walk a long time because you get tired quickly
4. You avoid walking whenever possible because it's too tiring for you

14. How do you feel about eating?
1. Just thinking about food makes you feel sick
2. You never enjoy eating
3. You are sometimes able to enjoy eating
4. You are always able to enjoy eating

15. To what extent do your treatments make your daily life more difficult?
1. Not at all
2. A little
3. Moderately
4. A lot

CFQ-UK

Adolescents and Adults (Patients 14 Years Old and Older)

CYSTIC FIBROSIS QUESTIONNAIRE - REVISED

16. How much time do you currently spend each day on your treatments?
1. A lot
2. Some
3. A little
4. Not very much

17. How difficult is it for you to do your treatments (including medications) each day?
1. Not at all
2. A little
3. Moderately
4. Very

18. How do you think your health is now?
1. Excellent
2. Good
3. Fair
4. Poor

Please select a box indicating your answer.

*Thinking about your health during the past **two weeks**, indicate the extent to which each sentence is true or false for you.*	Very true	Somewhat true	Somewhat false	Very false
19. I have trouble recovering after physical effort	☐	☐	☐	☐
20. I have to limit vigorous activities such as running or playing sports	☐	☐	☐	☐
21. I have to force myself to eat	☐	☐	☐	☐
22. I have to stay at home more than I want to	☐	☐	☐	☐
23. I feel comfortable discussing my illness with others	☐	☐	☐	☐
24. I think I am too thin	☐	☐	☐	☐
25. I think I look different from others my age	☐	☐	☐	☐
26. I feel bad about my physical appearance	☐	☐	☐	☐
27. People are afraid that I may be contagious	☐	☐	☐	☐
28. I get together with my friends a lot	☐	☐	☐	☐
29. I think my coughing bothers others	☐	☐	☐	☐
30. I feel comfortable going out at night	☐	☐	☐	☐
31. I often feel lonely	☐	☐	☐	☐
32. I feel healthy	☐	☐	☐	☐
33. It is difficult to make plans for the future (for example, going to college, getting married, getting promoted at work, etc.)	☐	☐	☐	☐
34. I lead a normal life	☐	☐	☐	☐

CFQ-UK

Adolescents and Adults (Patients 14 Years Old and Older)

CYSTIC FIBROSIS QUESTIONNAIRE - REVISED

Section III. School, Work, or Daily Activities

Questions 35 to 38 are about school, work, or other daily tasks.

35. To what extent did you have trouble keeping up with your schoolwork, professional work, or other daily activities during the past **two weeks**?

1. You have had no trouble keeping up
2. You have managed to keep up but it's been difficult
3. You have been behind
4. You have not been able to do these activities at all

36. How often were you absent from school, work, or unable to complete daily activities during the last two weeks because of your illness or treatments?

☐ Always ☐ Often ☐ Sometimes ☐ Never

37. How often does CF get in the way of meeting your school, work, or personal goals?

☐ Always ☐ Often ☐ Sometimes ☐ Never

38. How often does CF interfere with getting out of the house to run errands such as shopping or going to the bank?

☐ Always ☐ Often ☐ Sometimes ☐ Never

Section IV. Symptom Difficulties

Please select a box indicating your answer.

*Indicate how you have been feeling during the past **two weeks**.*	A great deal	Somewhat	A little	Not at all
39. Have you had trouble gaining weight?	☐	☐	☐	☐
40. Have you been congested?	☐	☐	☐	☐
41. Have you been coughing during the day?	☐	☐	☐	☐
42. Have you had to cough up mucus?	☐	☐	☐	☐

Go to Question 44

43. Has your mucus been mostly: ☐ Clear ☐ Clear to yellow ☐ Yellowish-green ☐ Green with traces of blood ☐ Don't know

*How often during the past **two weeks**:*	Always	Often	Sometimes	Never
44. Have you been wheezing?	☐	☐	☐	☐
45. Have you had trouble breathing?	☐	☐	☐	☐
46. Have you woken up during the night because you were coughing?	☐	☐	☐	☐
47. Have you had problems with wind?	☐	☐	☐	☐
48. Have you had diarrhoea?	☐	☐	☐	☐
49. Have you had abdominal pain?	☐	☐	☐	☐
50. Have you had eating problems?	☐	☐	☐	☐

Please make sure you have answered all the questions.

THANK YOU FOR YOUR COOPERATION!

Appendix 2: Consent form for Safety, efficacy and tolerability of Kalydeco in real-world setting: the experience of a single centre in the use of CFTR potentiator therapy

Clinical Research Ethics Committee Of The Cork Teaching Hospitals

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL

Section A

Protocol Number:_____ Patient Name:_____

_____

Title of Protocol: Safety, efficacy and tolerability of Kalydeco in real-world setting: the experience of a single-centre in the use of CFTR-potentiator therapy.

Doctor(s) Directing Research: Dr. Barry Plant Phone: (021) 4922327

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

Section B

I. NATURE AND DURATION OF PROCEDURE(S): Recent scientific studies show a health benefit to using Kalydeco as a treatment for people with CF with at least one copy of the G551D mutation. You have CF and have at least one copy of the G551D mutation, and you may benefit from taking this drug. Kalydeco has recently become available for use in Ireland, and will be paid for by the HSE subject to your participation in an ongoing program of assessment (including measurement of lung function, height, weight, sweat testing, and quality of life questionnaire) during the course of your treatment. The information from the scientific studies to-date shows that Kalydeco continues to work after 96 weeks of treatment without any significant safety concerns. However, there is no long-term information regarding the effects (positive or negative) or the safety profile of Kalydeco. We are undertaking a research study to address this. In order to better understand the effects of taking Kalydeco, you will be asked to donate a 20ml blood sample, a sputum sample and a stool (bowel motion) sample before starting Kalydeco therapy and then further samples for comparison after 3 months, 6 months and after 1 year. You will also be askedto undergo a specially adapted low-dose (equivalent of 3 chest x-rays) CAT scan of your lungs before starting Kalydeco with a repeat scan after 3-6 months of therapy. You will also be asked to perform exercise testing in the form of a standard 6-minute walk test (as per your annual assessment). Where possible these tests will be organized to take place on the same day as your routine, standard clinical assessments.

II. POTENTIAL RISKS AND BENEFITS: The risks to subjects will include the minor trauma and rare risks of bleeding and infections related to a standard blood test, and the exposure to radiation from a low-dose CAT scan. The precautions to minimize the risk associated with the blood test will be the use of standard universal precautions during blood sampling, and all blood samples will be taken by an experienced phlebotomist. The CAT scan will be especially adapted to ensure the lowest possible dose of radiation exposure, resulting in the same exposure as 3 chest x-rays. The information from the blood tests, sputum and stool samples will show us the effect of Kalydeco on your immune system, and its effect on the bugs that grow in your lung and your gut. The information from the CAT scan will allow us to see more clearly the effect of Kalydeco on specific areas of your lungs. This information may benefit you directly by allowing us to tailoryour future treatments according to the results of the tests. It may also benefit the next generation of potential Kalydeco users by providing a better understanding of the effects of Kalydeco on people with CF.

I. POSSIBLE ALTERNATIVES: Participation is voluntary. You may choose not to participate in this research study and you will still be eligible to receive Kalydeco subject to your participation in the HSE assessment program (as per Section B, Part I), and your treatment in the Cork Adult CF Centre will not be affected in any way.

Section C AGREEMENT TO CONSENT

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that ifI have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor: _____ _____

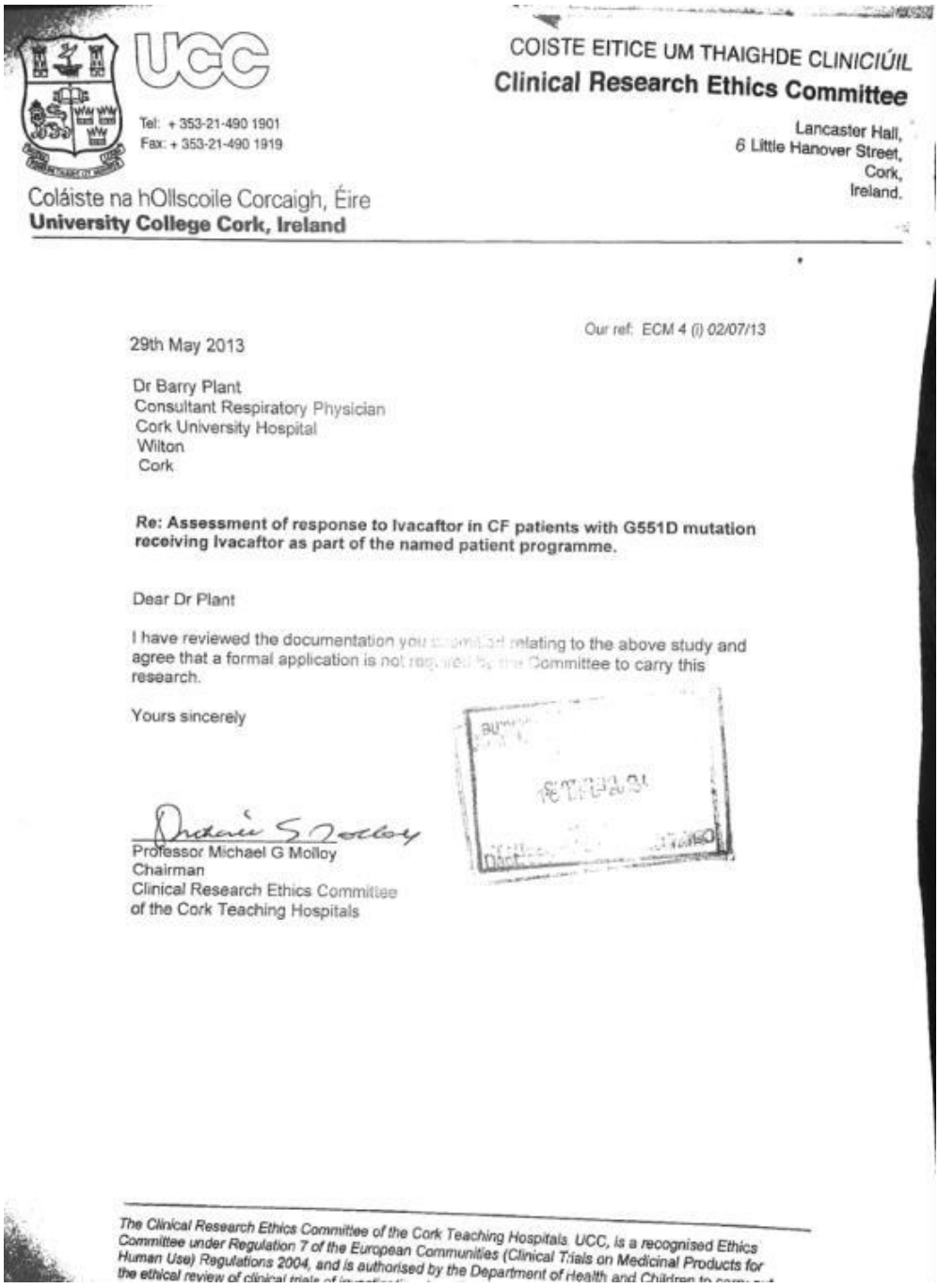
Signature of Subject, Parent or Guardian

(include a separate line for assent of minor, if applicable)

Witness:_____ Date:_____ Time:___ _____AM

(Circle) PM

Appendix 3: Letter of ethical approval for Safety, efficacy and tolerability of Kalydeco in real-world setting: the experience of a single centre in the use of CFTR potentiator therapy



UCC

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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee

Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Our ref: ECM 4 (i) 02/07/13

29th May 2013

Dr Barry Plant
Consultant Respiratory Physician
Cork University Hospital
Wilton
Cork

Re: Assessment of response to Ivacaftor in CF patients with G551D mutation receiving Ivacaftor as part of the named patient programme.

Dear Dr Plant

I have reviewed the documentation you submitted relating to the above study and agree that a formal application is not required by this Committee to carry this research.

Yours sincerely

Professor Michael G Molloy
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

The Clinical Research Ethics Committee of the Cork Teaching Hospitals UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised by the Department of Health and Children to carry out the ethical review of clinical trials of investigational

Appendix 4: Patient information leaflet Forskolin induced organoid swelling

Patient Information Leaflet

Protocol Title:

You are being asked to take part in a clinical research study that is being carried out in Cork Adult Cystic Fibrosis Centre. You may change your mind at any time for whatever reason and without having to justify your reason. Withdrawing from or not participating in the study will not have any negative impact on the care that you will receive from medical staff.

WHY IS THIS STUDY BEING DONE AND WHY AM I BEING ASKED TO PARTICIPATE?

Recent data have demonstrated that special cells "organoids" grown from rectal (bowel) biopsy samples from people with CF do not swell as much as those from people who do not have CF. Thus lack of organoid swelling can be helpful in diagnosing cystic fibrosis. Equally recent research has shown that Kalydeco (Ivacaftor) can enhance organoid swelling in rectal biopsy samples from patients with CF with certain mutation. Scientific studies show a health benefit to using Kalydeco as a treatment for people with CF with certain mutations including the G551D mutation. However, clinical response to treatment in some patients is variable. Worldwide approximately 5% of patients with CF carry the G551D mutation and can be treated with Kalydeco. In Cork 23% of patients have the G551D mutation and so can be treated with Kalydeco. The team in CUH and Utrecht want to investigate if the amount of organoid swelling observed in rectal biopsy samples of patients with CF currently taking Kalydeco mirrors their clinical improvement.

WHY IS THIS IMPORTANT TO FIND OUT?

This is an important question because it allows us to investigate if the amount of organoid swelling in patients on Kalydeco predicts the clinical improvement. If this is the case it might be possible to use this technology to identify patients with very rare mutations causing their CF who would benefit from medications such as Kalydeco. Also if organoids are shown to predict a patient's response to Kalydeco it may be possible to use organoids to investigate if other drugs would be useful in treating patients with CF.

HOW WILL THIS STUDY BE CARRIED OUT?

Consenting adult participants will be asked to give a rectal biopsy (sample of bowel lining) on a single occasion. This procedure can be done as an outpatient in the CF clinic. You will be awake during the procedure. You will be asked to lie on your side. A thin flexible tube will be placed in the rectum (back passage) and used to remove two or three tiny pieces of bowel. You should not experience any pain as there are no pain fibres in the rectum. The sample will be anonymised and transported to the Hubrecht Institute in the Netherlands where the organoids will be grown from the rectal biopsy and the amount of swelling measured. The amount of swelling will then be compared to your clinical improvement on

Kalydeco including change in lung function, weight, sweat chloride, Lung CAT scan and number of pulmonary exacerbations.

WHO IS ORGANISING THIS STUDY?

This study is being organised by Cystic Fibrosis Centre at CUH in collaboration with the research team in the Hubrecht Institute, Utrecht, Netherlands. The principal investigator is Professor Barry Plant, Consultant Respiratory Physician at the hospital.

POSSIBLE BENEFITS OF THE STUDY

This is an important question because if the amount of organoid swelling in patients on Kalydeco predicts the clinical improvement it might be possible to use this technology to identify patients with very rare mutations causing their CF who would benefit from medications such as Kalydeco. Also if organoids are shown to predict a patient's response to Kalydeco it may be possible to use organoids to investigate if other drugs would be useful in treating patients with CF.

KNOWN AND POTENTIAL RISKS ASSOCIATED WITH THE STUDY

Usually a rectal biopsy is a simple and safe procedure. This risks associated with this include a risk of bleeding. There is a small risk of developing an infection at the biopsy site.

CONFIDENTIALITY ISSUES

All data will be anonymised, password protected and stored on a secure computer in the respiratory and radiology departments to which only the research team will have access.

Appendix 5: Consent form for FIS study

Clinical Research Ethics Committee Of The Cork Teaching Hospitals

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH PROTOCOL

Section A

Protocol Number:_____ Patient Name:_____

Title of Protocol:

Doctor(s) Directing Research: Prof. Barry Plant Phone: (021) 4922327

You are being asked to participate in a research study. The doctors at University College Cork study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgment. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

Section B

NATURE AND DURATION OF PROCEDURE(S): You are being asked to participate in a research study. The CF Team at Cork University Hospital in collaboration with scientist in the Hubrecht Institute, Utrecht, Netherlands study the nature of disease and are attempting to understand if special cells "Organoids" grown from rectal biopsy samples of patients with CF can predict a patients clinical response to current CF mutation specific medications such as Ivacaftor (Kalydeco). Recent data have demonstrated that organoids from people with CF do not swell as much as those from people who do not have CF. Equally recent research has shown that Kalydeco can enhance organoid swelling in rectal biopsy (bowel lining) samples from patients with CF with certain mutation. Scientific studies show a health benefit to using Kalydeco as a treatment for people with CF with certain mutations. However, clinical response to treatment in some patients is variable. The team in CUHand Utrecht want to investigate if the amount of organoid swelling observed in rectal biopsy samples of patients with CF treated with Kalydeco can predict their clinical improvement. If you choose to participate a rectalbiopsy will be performed as an outpatient. You will be awake during the procedure. You will be asked to lie on your side. A thin flexible tube will be placed in the rectum (back passage) and used to remove two or three tiny pieces of bowel. You should not experience any pain as there are no pain fibres in the rectum.

I. POTENTIAL RISKS AND BENEFITS: Your participation may help us to better understand if the amount of organoid swelling can predict a patient's response to treatment with CFTR modulatory medications such as Kalydeco. This may be of benefit to patients with CF with very rare mutation in which it is not possible to perform large drug studies. Similarly if organoid swelling predicts patients response to Kalydeco it may be possible to test other drugs on organoids from patients with CF and identify new treatments for patients with CF or identify a drug that may be more beneficial to individual patients. The potential risks include those relating to rectal biopsy which includes bleeding at the biopsy site and risk of infection. To minimise the risk of breach of confidentiality all samples will be anonymized and data will be stored in a secure location to which only members of the CF team will have access.

II. POSSIBLE ALTERNATIVES: You may change your mind at any time for whatever reason and without having to justify your reason. Withdrawing from or not participating in the study will not have any negative impact on the care that you will receive from medical staff.

Section C AGREEMENT TO CONSENT

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I

have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, the records of this research may be reviewed by government agencies and sponsors of the research.

I understand that the sponsors and investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at the Cork Teaching Hospitals. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor(s) listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

CF Team Member: _____

Role: _____

Signature of Subject, Parent or Guardian_____

Signature of Patient_____

Date:_____ Time:_____AM
(Circle) PM

Appendix 6: FIS Ethical approval letter

UCC
Tel: + 353-21-490 1901
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Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee
Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Our ref: ECM 4 (g) 19/01/16

7th January 2016

Professor Barry Plant
Director Cork Adult Cystic Fibrosis Centre
Cork University Hospital
Wilton
Cork

Re: The relationship between forskolin induced intestinal organoid swelling and clinical response in patients with cystic fibrosis receiving ivacaftor therapy.

Dear Professor Plant

Expedited approval is granted to carry out the above study at:

- Cork University Hospital.

The following documents have been approved:

- Application Form signed 14th December 2015
- Study Protocol Version 1.0 dated 14th December 2015
- Patient Information Leaflet Version 1 dated 14th December 2015
- Consent Form Version 1.0 dated 14th December 2015
- New Adult CF Patients Version 1.0 dated 14th December 2015
- Cover Letter dated 14th December 2015.

The co-investigators involved in the study will be:

- Dr Nicola Ronan, Research Registrar, Ms Mary Daly, Research Nurse, Dr Akbar Consultant Gastroenterologist, Dr Evelyn Flanagan, Data Manager and Ms Yvonne McCarthy, CF Scientist.

Yours sincerely

Professor Michael G Molloy
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

The Clinical Research Ethics Committee of the Cork Teaching Hospitals, UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised by the Department of Health and Children to carry out the ethical review of clinical trials of investigational medicinal products. The Committee is fully compliant with the Regulations as they relate to Ethics Committees and the conditions and principles of Good

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