UNIVERSIDADE D
COIMBRA

Juliana Loureiro Fidalgo Roda

NOVEL PERSONALIZED THERAPIES FOR CYSTIC FIBROSIS IN A PAEDIATRIC POPULATION

Tese no âmbito do Programa de Doutoramento em Ciências da Saúde, ramo de Medicina, orientada pela Professora Doutora Guiomar Gonçalves de Oliveira e pela Professora Doutora Margarida Duarte Amaral e apresentada à Faculdade de Medicina da Universidade de Coimbra.

Dezembro de 2022

Faculdade de Medicina da Universidade de Coimbra

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The studies presented in this thesis were carried out at Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra and at BioISI - Biosystems & Integrative Sciences Institute, Faculty of Sciences of the University of Lisbon.

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The present study was approved by the board of the Centro Hospitalar e Universitário de Coimbra after a favourable report by the Health Ethics Committee (Ref. CHUC-080-16).

Informed consent to participate was obtained from all parents, as all subjects that participated in the study are under 18 years old, and also from the subjects with Cystic Fibrosis themselves with 16 years old or older.

Informed consent for publication was obtained from all parents, as all subjects are under 18 years old, and also from the subjects with Cystic Fibrosis themselves with 16 years old or older.

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List of publications

Results used in this dissertation were included in the following articles:

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- Roda J, Maia C, Almeida S, Oliveira RC, Ferreira R, Oliveira G. Faecal calprotectin and rectal histological inflammatory markers in cystic fibrosis: a single-centre study. BMJ Paediatrics Open 2022; 6: e001422. doi: 10.1136/bmjpo-2022-001422
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- Roda J, Pinto-Silva C, Silva IAL, Maia C, Almeida S, Ferreira R, Oliveira G. New drugs in cystic fibrosis: what has changed in the last decade? Ther Adv Chronic Dis. 2022;13:20406223221098136. Published 2022 May 21. doi:10.1177/20406223221098136
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- **Intestinal Organoids as a Good Tool to Predict Pre-clinical Response to Cystic Fibrosis Transmembrane Conductance Regulator Modulators: a Small Portuguese Cohort**

  Juliana Roda, Iris Silva, Violeta Railean, Cláudia Rodrigues, Susana Almeida, Carla Maia, Ricardo Ferreira, Guiomar Oliveira, Margarida D Amaral

  Oral communication. XXXIV Annual Meeting of the Portuguese Society of Paediatric Gastroenterology, Hepatology and Nutrition. Lisbon, Portugal. 5-6 May 2022.
- **Intestinal Organoids for the Personalized Treatment of Cystic Fibrosis**

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  Oral communication. 6th World Congress of Paediatric Gastroenterology, Hepatology and Nutrition, Vienna, Austria. 4 June 2021.
- **Faecal calprotectin evaluation in paediatric patients with Cystic Fibrosis**

  Cláudia Arriaga, Juliana Roda, Carla Maia, Susana Almeida, Ricardo Ferreira

  Oral communication. XXXIII Annual Meeting of the Portuguese Society of Paediatric Gastroenterology, Hepatology and Nutrition. Amarante, Portugal. 25-26 November 2021.
- **Importance of bone density evaluation in paediatric patients with Cystic Fibrosis**

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- **Vitamin E supplementation with fat soluble formulation in children with Cystic Fibrosis: two years' follow-up.**

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Invited Speaker

- **Phisiopathology of Cystic Fibrosis**
  III Faculty of Pharmacy of the University of Coimbra -Talks. Coimbra, Portugal. 18 December 2021.
- **Nutritional management in special situations: Cystic Fibrosis**
  Fall School of Annual Meeting of the Portuguese Society of Paediatric Gastroenterology, Hepatology and Nutrition. Pedrogão Pequeno, Portugal. 24-26 October 2019.
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Resumo

A Fibrose Quística (FQ) é a doença autossómica recessiva limitante da vida mais comum em caucasianos. Esta doença genética é causada por variantes no gene regulador da condutância transmembranar da FQ (*CFTR*) que codifica um canal de cloreto (Cl^-) na membrana plasmática apical das células epiteliais. Variantes no gene *CFTR* originam secreções anormalmente viscosas responsáveis por uma grande variabilidade de manifestações clínicas em diferentes órgãos. Novos medicamentos que visam variantes específicas do gene *CFTR* já entraram na prática clínica, mas não se aplicam às mais de 2,000 variantes descritas, excluindo indivíduos com variantes raras. Estes moduladores de CFTR estão divididos em dois grupos: potenciadores (Ivacaftor) e corretores (Lumacaftor, Tezacaftor e Elexacaftor).

Este trabalho teve como objetivo principal a caracterização clínica e genética de uma coorte de indivíduos com FQ para uma potencial intervenção terapêutica personalizada com moduladores de CFTR utilizando organoides intestinais.

Foi realizada a caracterização clínica e genética de uma coorte de 23 pessoas com FQ (pwCF) em idade pediátrica seguidos no Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra. Todos os indivíduos têm a variante p.Phe508del em pelo menos um alelo. Quinze pwCF p.Phe508del-homozigóticos são elegíveis para terapia dupla (Luma/Teza+Iva) e para terapia tripla (Teza+Iva+Elexa). As pwCF com as variantes de função mínima c.579+1G>T (n=2), p.Gln685ThrfsX4 (n=1) e c.3321dup (n=1) são elegíveis para terapia tripla. Os doentes com uma variante com função residual: p.Arg334Trp (n=3) e p.Pro5Leu (n=1) têm um fenótipo menos grave, no entanto, em Portugal, não têm atualmente nenhuma terapia disponível. A caracterização genética e molecular de pwCF representa um passo importante não apenas para o diagnóstico e prognóstico da FQ, que está fortemente relacionado com o fenótipo clínico, mas também para a elegibilidade de medicamentos moduladores de CFTR.

Nesta coorte de 23 pwCF, foi também analisada a associação entre as características clínicas e o valor de calprotectina fecal (um marcador de inflamação intestinal). Dezassete (17/23) pwCF apresentavam calprotectina elevada e o valor mediano foi de 88 µg/g

(IQR=17µg/g). Nos indivíduos com insuficiência pancreática (IP) foi observada calprotectina mais elevada (101*vs*30µg/g, P=0.027). Não foi encontrada associação entre calprotectina elevada e o estado nutricional ou a presença de sintomas digestivos. Em 11 destes indivíduos, foram obtidas biópsias retais e foi encontrada inflamação retal focal inespecífica nas amostras de quatro (4/11). Este achado apresentou uma associação significativa com o valor elevado da calprotectina (p=0.015). A sensibilidade foi de 100% e a especificidade de 86%. Nesta coorte de pwCF, a elevação da calprotectina fecal foi frequente, principalmente nos com IP, e estava presente nos indivíduos com evidência histológica de inflamação retal. A calprotectina fecal pode ser um indicador de inflamação retal assintomática em indivíduos com FQ.

As biópsias retais dos mesmos 11 indivíduos foram utilizadas para determinar a função residual de CFTR e para prever respostas aos fármacos moduladores de CFTR. A secreção de Cl- mediada por CFTR foi avaliada por medições de voltagem intestinal (IVM) em câmara de Ussing. Paralelamente, os organoides intestinais foram preparados, cultivados e analisados pelo ensaio de Inchamento Induzido por Forscolina (FIS) com microscopia confocal, antes e após incubação com os moduladores, nomeadamente: Iva, Teza/Iva ou Elexa/Teza/Iva. Os oito pwCF com variantes raras e p.Phe508del no outro alelo foram incluídos: três com p.Arg334Trp e um com p.Pro5Leu (que têm um fenótipo atípico e, efetivamente, exibem função residual CFTR em ambos os ensaios IVM e FIS); dois com c.579+1G>T, um com p.Gln685ThrfsX4 e um com a variante não descrita anteriormente c.3321dup (que têm um fenótipo CF clássico e nenhuma função CFTR no ensaio IVM e FIS). Três pwCF p.Phe508del- homozigóticos também participaram do estudo, dois não apresentavam função residual de CFTR (no terceiro os resultados foram inconclusivos). A proteína CFTR foi resgatada por Teza/Iva em organoides com variantes p.Arg334Trp e p.Pro5Leu. No entanto, esta combinação falhou em resgatar a função CFTR nos organoides p.Phe508del-homozigóticos e nos organoides com as variantes c.579+1G>T, p.Gln685ThrfsX4, c.3321dup. A terapia tripla Elexa/Teza/Iva resgatou significativamente a proteína CFTR nos organoides de cinco pwCF: dois com a variante c.579+1G>T, um com p.Arg334Trp e dois p.Phe508del-homozigóticos. Estes resultados evidenciam o interesse da utilização de organoides intestinais na previsão da resposta individual aos moduladoras de CFTR, indicando que pwCF com as variantes p.Arg334Trp ou p.Pro5Leu podem beneficiar do tratamento com Teza/Iva. Uma resposta

clínica mais significativa é esperada para a terapia tripla em pwCF com a variante p.Phe508del em pelo menos um alelo.

Abstract

Cystic Fibrosis (CF) is the most common life-limiting autosomal recessive disease among Caucasians. This genetic disease is caused by variants in the CF transmembrane conductance regulator (*CFTR*) gene which encodes a chloride (Cl^-) channel in the apical plasma membrane of epithelial cells. Variants in the *CFTR* gene cause abnormally viscous secretions responsible for a wide variability of clinical manifestation in different organs. New approved drugs that target specific gene *CFTR* variants have entered clinical practice but do not apply to all of the 2,000 CFTR variants reported, excluding individuals with rare variants. These CFTR modulators are divided in two main groups: potentiators (Ivacaftor) and correctors (Lumacaftor, Tezacaftor and Elexacaftor). The main objective of this doctoral work is to characterize clinically and genetically a small paediatric cohort of individuals with CF for a potential personalized therapeutic intervention with CFTR modulators using intestinal organoids.

A cohort of 23 paediatric people with CF (pwCF) undergoing follow-up at Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra had their clinical and genetic features analysed in order to determine which ones are candidates to CFTR modulators. All individuals have the p.Phe508del variant in at least one allele. Fifteen pwCF were p.Phe508del-homozygous and are eligible for dual therapy (Luma/Teza+Iva) and for triple therapy (Teza+Iva+Elexa). pwCF with c.579+1G>T (n=2), p.Gln685ThrfsX4 (n=1) variants and a novel variant c.3321dup (n=1) have minimal function variant and a classic phenotype which also makes them eligible to triple therapy. Patients with a residual function variant: p.Arg334Trp (n= 3) and p.Pro5Leu (n=1) have a less severe phenotype, however in Portugal, they have at present no available therapy. Genetic and molecular characterization of pwCF poses an important step not just for CF diagnosis and prognosis which is tightly correlated with the clinical phenotype, but also for the eligibility of CFTR modulator drugs.

In this cohort of 23 paediatric pwCF the association of faecal calprotectin (a biomarker of intestinal inflammation) with the clinical characteristics was analysed. Seventeen (17/23) pwCF had elevated faecal calprotectin, and the median value was 88µg/g (IQR=178µg/g). Higher faecal calprotectin levels were observed in the pancreatic

insufficient (PI) group (101*vs*30 µg/g, P=0.027). No significant association between elevated faecal calprotectin and nutritional status or gastrointestinal symptoms was found. In 11 of these individuals, rectal biopsies were obtained and unspecific focal rectal inflammation was found in four individuals (4/11). This finding was associated with elevated faecal calprotectin (p=0.015). Sensitivity was 100% and specificity was 86%. In pwCF analysed here, elevated faecal calprotectin was frequent, particularly if PI, and it was present in individuals with histologic evidence of rectal inflammation. Faecal calprotectin may be an indicator of asymptomatic rectal inflammation in pwCF.

Rectal biopsies from the same 11 pwCF were used in order to determine CFTR residual function and to predict specific responses to approved CFTR modulator drugs. CFTR-mediated Cl^- secretion was assessed by Ussing chamber intestinal voltage measurements (IVM). In parallel, intestinal organoids were prepared, cultured and analysed using the Forskolin-Induced Swelling (FIS) assay by confocal microscopy, before and after incubation with modulators, namely: Iva, Teza/Iva or Elexa/Teza/Iva. The eight pwCF with rare variants and p.Phe508del in the other allele, were included: three with p.Arg334Trp and one with p.Pro5Leu (who have an atypical phenotype and in fact, exhibit CFTR residual function in both IVM and FIS assays); two with c.579+1G>T, one with p.Gln685ThrfsX4 and one with the variant c.3321dup (who have a classical CF phenotype and no CFTR function in IVM and FIS assays). Three pwCF who were homozygous for p.Phe508del with variable clinical phenotype also participated, two had no residual CFTR function (and one with no conclusive analysis). CFTR was rescued by Teza/Iva in organoids with p.Arg334Trp and p.Pro5Leu variants. However, this combination failed to rescue CFTR function in c.579+1G>T, p.Gln685ThrfsX4, c.3321dup and p.Phe508del-homozygous organoids. Triple therapy Elexa/Teza/Iva significantly rescued CFTR in organoids from five pwCF: two with c.579+1G>T, one with p.Arg334Trp and two p.Phe508del -homozygous. Our results illustrate the value of using intestinal organoids to predict individual responses to approved CFTR modulator drugs, indicating that individuals with CF bearing p.Arg334Trp or p.Pro5Leu variants may benefit from treatment with double therapy of CFTR modulators. A more significant clinical benefit is expected for the triple therapy in pwCF carrying the p.Phe508del variant in at least one allele.

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List of abbreviations

Amil	Amiloride
ATP	Adenosine triphosphate
AUC	Area under the curve
BCC	*Burkholderia cepacian complex*
BMD	Bone mineral density
BMI	Body mass index
Ca^{2+}	Calcium ion
CaCC	Ca^{2+}-activated alternative Cl^- channels
cAMP	Cyclic adenosine monophosphate
CBAVD	Congenital bilateral absence of vas deferens
CCH	Carbachol
CF	Cystic Fibrosis
CFBD	CF-related bone disease
CFBE	CF bronchial epithelial
CFF	Cystic Fibrosis Foundation (USA)
CFLD	CF-related liver disease
CFRD	CF-related diabetes
CFTR	CF transmembrane conductance regulator
Cl^-	Chloride ion
CT	Computerized tomography (scan)
DEXA	Dual energy X-ray absorptiometry
DIOS	Distal intestinal obstruction syndrome
DNA	Deoxyribonucleic acid
E/T/I	Elexacaftor/Tezacaftor/Ivacaftor
ECFS	European Cystic Fibrosis Society
Elexa	Elexacaftor
EMA	European Medicines Agency
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
EU	European Union

FEV1	Forced expiratory volume in one second
FIS	Forskolin induced swelling (assay)
FRT	Fischer rat thyroid (cells)
Fsk	Forskolin
FVC	Forced vital capacity
HBE	Human bronchial airway epithelial cells
$HCO3^-$	Bicarbonate ion
HE4	Human epididymis protein 4
HEMT	Highly effective CFTR modulator therapy
HNE	Human nasal epithelial
HP-CHUC	Hospital Pediátrico – Centro Hospitalar Universitário de Coimbra
IBMX	3-isobutyl-1-methylxantine
IC/VM	Intestinal current/voltage measurement
ICM	Intestinal current measurement
IL	Interleukin
Indels	(small gene) insertions or deletions
IQR	Interquartile range
Infarmed	Autoridade Nacional do Medicamento e Produtos de Saúde, I.P.
IRT	Immunoreactive trypsinogen
Iva	Ivacaftor
IVM	Intestinal voltage measurement
K^+	Potassium ion
kb	Kilobases
kDa	Kilodalton
Luma	Lumacaftor
Luma/Iva	Lumacaftor/Ivacaftor
NBS	Newborn screening
MCC	Mucociliary clearance
MVCC	Variants of varying clinical consequences
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
NBD1/2	Nucleotide binding domain 1/2

NBS	Newborn screening
NE	Neutrophil elastase
NF-KB	Nuclear factor kappa of activated B cells
NHS	National health service
NPD	Nasal potential difference
Pa	*Pseudomonas aeruginosa*
PAP	Pancreatitis associated protein
PERT	pancreatic enzyme replacement therapy
pH	Potential of hydrogen
PI	Pancreatic insufficiency
PM	plasma membrane
PS	Pancreatic sufficient
PTC	Premature termination codons
pwCF	Person/people with Cystic Fibrosis
q	Long arm of the chromosome
RD	Regulatory domain
R_{te}	Transepithelial resistance
SCC	Sweat chloride concentration
SCT	Sweat chloride test
Teza	Tezacaftor
Teza/Iva	Tezacaftor/Ivacaftor
TMD 1/2	Transmembrane domain 1/2
TNF-α	Tumor necrosis factor α
UPP	Ubiquitin-proteasome pathway
US	United States of America
V_{te}	transepithelial voltage
VX-445	Elexacaftor
VX-661	Tezacaftor
VX-770	Ivacaftor
VX-809	Lumacaftor
wt	Wild type

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Chapter 1. General Introduction

Part I – Introduction

Cystic Fibrosis (CF) is the most common life-limiting autosomal recessive disease among Caucasians [1]. This genetic disease is caused by variants in the CF transmembrane conductance regulator (*CFTR*) gene which encodes a chloride (Cl^-) channel in the apical plasma membrane (PM) of epithelial cells. Variants in the *CFTR* gene cause abnormally viscous secretions in the cells of diferent epithelia. Despite the wide clinical variability in organ involvement, CF predominantly affects mostly the lungs and respiratory tract, but also the pancreas, liver, sweat glands, intestinal tract and commonly leads to congenital bilateral absence of *vas deferens* (CBAVD) [2].

Historic note

The first suspected reference to the disease is in an Irish proverb from the end of the fifteenth century, which says: "*Woe to the child who tastes salty when kissed on the forehead. He is bewitched and soon must die*" [3]. Some isolated case reports of children who died of diarrhea and malnutrion and with the presence of hardened pancreas as a common denominator in the autopsy, are found since 1595 [3].

However, it was not until 1938 that CF was first described by Dorothy Andersen, based on the histologic finding of autopsies of children who died of malnutrition, where the term "cystic fibrosis of the pancreas" was first used [3].

In 1945, Sydney Farber speculated that CF was not only a localized pancreatic problem but a generalized impairment of mucus production, coining the term ''mucoviscidosis''. The following year, the aforementioned Dorothy Andersen conducted studies on relatives of people with CF (pwCF) and arrived to the conclusion that this was a genetic disease with autosomal recessive inheritance pattern [3].

Lung infection and electrolyte disturbances were later associated to the disease during a heat wave in New York in August 1948, where many children with CF developed severe hyponatremic dehydration [4]. Years later (1953), Di Sant´Agnese demonstrated that excessive salt loss was found in the sweat of pwCF. The technique for measuring salt loss was improved by Gibson and Cooke (1959), resulting in the sweat test still used today as the gold standard tool for the diagnosis of CF [3,4].

In the 1950´s , most pwCF died before the age of one year. The main causes of death were a severe intestinal obstruction at birth, called meconium ileus, and malnutrition subsequent to pancreatic insuficiency (PI), maldigestion and consequent malabsorption [2]. Studies on pancreatic enzyme therapy had been described in 1900 to reduce excessive faecal fat and nitrogen loss. An important breakthrough came in 1958, when the Canadian clinician Douglas Crozier gave his patients a diet rich in saturated fats, completely contrary to what had been done up until then, and high doses of oral pancreatic enzyme supplements thereby managing to improve nutritional status and growth and increase survival [3]. On this same year the first commercial pancreatic enzyme preparation obtained from bovine and porcine extracts was approved for clinical use: pancreatin (Pankreon®) [3,4].

In 1981, Knowles *et al* demonstrated the presence of an abnormally increased potential difference across the nasal mucosa that resulted from an abnormal epithelial function. This led Quinton *et al* to study the problem in the sweat duct. He demonstrated that impermeability to chloride meant that this could not be reabsorbed by the blood. This finding, applied to other epithelia, would explain the viscosity of mucus in these individuals [3].

The gene responsible for CF was mapped in 1985 to chromosome 7 and the real explosion of knowledge came out in 1989 with the identification of the *CFTR* gene [3,5]. Sequencing comparison between alleles from individuals and their parents revealed the most common CF gene variant, a deletion causing loss of phenylalanine at position 508 of the protein, designated formerly Δ*F508* [3], afterwards designated as F508del and at present p.Phe508del. In 1990, Welsch *et al* demonstrated a causal relationship between variants in the *CFTR* gene and defective Cl^- ion transport [3]. The major goal of identifying the CF gene was to be able to move quickly to gene therapy. The concept of giving individuals a correct copy of the CF gene, so that they could produce the functional protein, seemed simple. However, gene therapy trials soon demonstrated how difficult it is to deliver and express foreign genes in some organs and soon enthusiasm about this approach faded [5]. Since then, a large variety of *CFTR* gene variants has been described. However, determining genotype-phenotype correlations proved to be difficult and the next challenge in the CF field was to identify the molecular and cellular dysfunction caused

by these different gene variants. Advances in fundamental science have provided a better understanding of the mechanisms of protein folding, pathways of secretory traffic (both impaired in the most common mutant protein, p.Phe508del-CFTR) and the phisiology of epithelial channels, making a paradigm for traficking disorders and other "chanelopathies". Later on, the generation of CF animal models led the field of animal disease models through the application of gene targeting therapy. This generation of CF mouse models involved the three 2007 Nobel Prize awardees in this field [5].

Meanwhile, further advances in airway clearance and aggressive treatment of pulmonary infections became available, based in inhaled mucolytics to loosen and clear mucus characteristic of CF, chest phisiotherapy and nebulized, oral and intravenous antibiotics. The widespread of lung transplant, first performed in a pwCF in 1985, further contibuted to increase life expectancy of these individuals [3].

Despite all the advances in basic science and clinical management of CF in 2009, when celebrating the 20th anniversary of the discovering of the gene, median survival was still in the third decade[5]. This lead to John Riordan, who together with Lap-Chee Tsui and Francis Collins made the original *CFTR* gene discovery, to *state "The disease has contributed much more to science than science has contributed to the disease"*[6].

During the past six decades, a disease that was fatal in the first year of life, has now a median age of survival of more than 40 years old in some developed countries and in December 2022, it was announced by the US Cystic Fibrosis Foundation (CFF) that life expectancy for a child born now is of 53 years [2].

Epidemiology and demographics

Eventhough, CF is the most common genetic life-limiting disease in the caucasian population it is classified as a rare disease, afecting more than 90,000 individuals worldwide and over 40,000 in Europe [4,7,8]. The estimated incidence of CF worldwide is 1 in 2,500-4,000 newborns [5] with a recognized heterogeneity in the geographic distribution. Incidence is higher in Europe, North America and Australia (1/3,000), followed by the Middle East (Israel: Ashkenazi Jews and Arabs 1/1,800-4,000; Bahrain

1/6,000), South America (Brasil 1/7,000), África (South Africa 1/12000) and Asia (Japan 1/350,000) [9]. Estimated prevalence of CF in Europe is 0.74 per 10,000 inhabitants [10].

In Portugal, recent data based in newborn screening (NBS) results reported an incidence of 1 in 7,500 newborns[11]. Prevalence is lower in comparison to the rest of Europe, estimated in 0.27 per 10,000 inhabitants [10]. In absolute numbers, there are 327 individuals with CF followed in Portuguese CF reference centres [8].

CF is a monogenic disorder with a recessive autosomal hereditary pattern, this means that two alleles with pathogenic variants are needed for the disease to manifest. The estimated frequency of CF variant carriers is 1/25 individuals with Northern-European background [12]. The high incidence of CF carriers is explained by some authors as a protective effect or advantage for carriers of *CFTR* variants. For example, one report proposed that it is CFTR protein that mediates the translocation of *Salmonella typhi* into de gastrointestinal submucosa. Thus, people with one CFTR variant are expected to have increased resistance to typhoid fever [13].

The characteristics of the CF population have changed substantially over the last four decades. One of the most dramatic changes observed has been the growing adult population, which is over 50% of pwCF in most developed countries. This growth is largely driven by more paediatric pwCF surviving to adulthood, as opposed to an increasing number of adult diagnosis. In the 1960s, CF was almost exclusively a paediatric disease with a median survival less than five of age. In 1990, the median age of US CF population was 12.5 years with 33 % of pwCF over the age of 18 years. In 2014, this percentage increased to over half of the pwCF worlwide [14] and is now 51% in Europe [8]. These facts are due to the lower CF-related paediatric mortality in developed countries and the markedly increased longevity in adults with CF [14].

It is expected that, in developed European countries, the percentage of adults with CF will increase by around 70% by 2025 [2].

Even though, the number of adults with CF continues to increase, while the number of children remains relatively stable, the median age of pwCF currently in the European Registry is 18.5 years and the median age at death is 32.4 years [8].

Pathophysiology

CFTR gene

CF is a classic mendelian autosomal recessive disorder resulting from variants in a gene located on the long arm (q) of chromosome seven. The gene comprises 27 coding exons, spanning over 190 kilobases (kb) of human genomic deoxyribonucleic acid (DNA) [15].

The most common CF-causing variant is p.Phe508del [13]. Around 40% of all European individuals with CF are homozygous for this variant and another 35-40% are heterozygous [16].

Over 2,100 variants have been identified along the entire CFTR gene of which 485 have been annotated in the CFTR2 Mutation Database (as of 29 April 2022): 401 are confirmed CF-causing; 49 have variable clinical consequences; 24 are non CF-causing (or neutral) and 11 are of unknown significance. Many other variants are still uncharacterized [17].

In most countries, only 10 to 15 variants occur at a frequency above 1%. Many *CFTR* variants are rare or very rare, only occurring in few on even a single person [18]. In fact, more than 1,000 variants occur in less than 5 families worldwide.

The different *CFTR* gene variants can be classically divided in six major functional classes according to their effect on CFTR protein function and in which the same restorative strategy may be effective [5]. More recently, a seventh class has been proposed [19] (Figure 1).

Class I: Defective protein synthesis (no protein)

Variants in this class include nonsense variants, i.e., those introducing a premature termination codon (PTC). PTCs are known to result in truncated proteins that are rapidly degraded. Therefore, such variants are expected to produce no protein. This class includes p.Gly542X (legacy name: G542X) and p.Trp1282X (legacy name: W1282X) variants [5,13].

Class II: Defective protein processing (no trafficking)

After translation, the normal protein undergoes folding and core-glycosylation (immature form) in the endoplasmic reticulum (ER) and after it proceeds its traffic through the Golgi apparatus to reach the apical PM, the protein acquires its fully-glycosylation pattern (mature form). Class II variants cause impairment of this process, which leads to premature degradation of the abnormally processed protein. The most common gene variant, p.Phe508del, is a paradigmatic example of this variant class. It results in a CFTR protein that is unable to correctly fold into its appropriate 3D-conformation, being consequently retained and degraded by the ER quality control (97% *versus* 75% in normal proteins) [5,13].

Class III: defective protein function (no function)

CFTR activity is regulated by phosphorylation and dephosphorylation. In addition, the normal gating (both opening and closing) cycle of the Cl^- channel requires adenosine triphosphate (ATP) binding and hydrolysis. Proteins from Class III variants, although reaching the PM, exhibit defective channel gating (the channel pore does not open). An example of this class is p.Gly551Asp variant (legacy name: G551D) [5,13].

Class IV: altered protein conductance (less function)

These variants are associated with reduced flow of ions through the CFTR channel pore, corresponding to a reduced rate of Cl^- transport. The p.Arg334Trp variant (legacy name: R334W) is an example [5,13].

Class V: reduced CFTR levels (less protein)

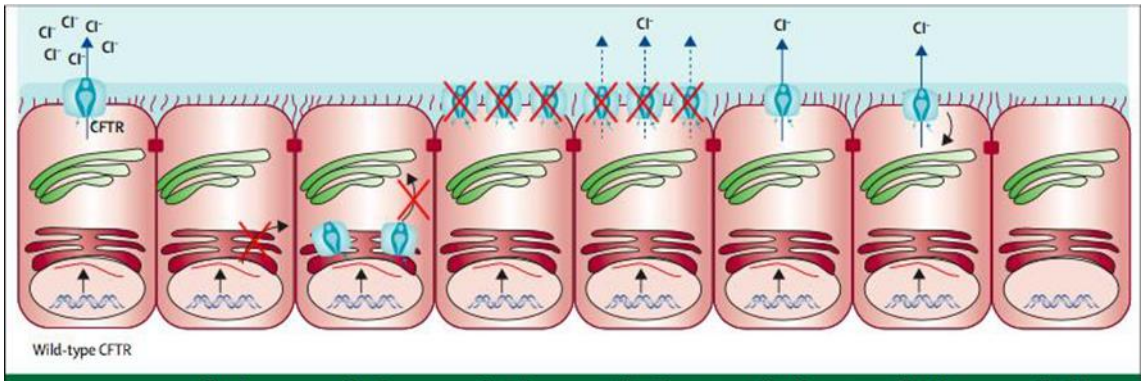
These cases lead to the production of normal protein in less quantity. It includes variants that reduce transcription and amino acid substitutions that cause inefficient protein maturation (e.g., p.Ala455Glu, legacy name: p.Ala455Glu). Yet, most of the variants are splicing variants that allow synthesis of some normal CFTR protein, albeit at a very low levels (e.g., c.3140-26A>G, legacy name: 3272-26A->G) [5,13].

Class VI: decreased PM stability (accelerated turnover)

These class impair the CFTR PM stability. Nonsense and frameshift variants cause increased turnover of the CFTR protein at the PM. Such abnormalities do not impair the biogenesis of CFTR, but are often caused by a C-terminus truncation, leading to degradation of the mature CFTR protein at a rate that is 5 to 6 times faster than normal[5,20].

Class VII: no mRNA transcription

More recently, a seventh class has been proposed by De Boeck and Amaral [19]. They divided the traditional class I variants into class I (stop-codon variants) and a new class VII comprising unrescuable variants such as frameshifts due to insertions or deletions. Class VII variants have the same outcome as the class I (absence of the CFTR protein) —but cannot be rescued by corrective PTC therapy [19]. This classification was previously suggested by other authors as division of Class I in two branches: class IA and class IB [21].



	Class I	Class II	Class III	Class IV	Class V	Class VI	Class VII
CFTR defect	No protein	No traffic	Impaired gating	Decreased conductance	Less protein	Less stable	No mRNA
Mutation examples	Gly542X, Trp1282X	Phe508del, Asn1303Lys, Ala561Glu	Gly551Asp, Ser549Arg, Gly1349Asp	Arg117His, Arg334Trp, Ala455Glu	Ala455Glu, 3272-26A→G, 3849+10 kg C→T	c. 120del23, rPhe508del	dele2,3(21 kb), 1717-1G→A
Corrective therapy	Rescue synthesis	Rescue traffic	Restore channel activity	Restore channel activity	Correct splicing	Promote stability	Unrescuable
Drug (approved)	Read-through compounds (no)	Correctors (yes)	Potentiators (yes)	Potentiators (no)	Antisense oligonucleotides, correctors, potentiators? (no)	Stabilisers (no)	Bypass therapies (no)

Figure 1.1. Functional classification of CFTR variants and theratype-specific CFTR modulators.
CFTR variants can be grouped into seven classes (I-VII), according to the functional defect elicited on the CFTR protein. Classes' I-III and VII variants with absence or severe loss of CFTR production and function by different mechanisms and are associated with no residual function. Classes IV-VI variants are less severe and associated with some residual, although highly variable, CFTR function. Functional defect-specific corrective therapeutic approaches have been proposed to be applicable to all variants belonging to the same class (theratypes). Variant examples are presented. Gly = Glycine; X = Stop codon; Trp = Trypthophan; Phe = Phenylalanine; del = deletion; Asn = Asparagine; Lys = Lysine; Ala = Alanine; Glu = Glutamine; Asp = Aspartate; Ser = Serine; Arg = Arginine; His = Histidine; r = rescued.

Retrieved from The Lancet Respiratory Medicine,Volume 4, Issue 8, Kris De Boeck, Margarida D Amaral, Progress in therapies for cystic fibrosis, Pages 662-674, (2016), with permission from Elsevier [19].

This classification approach is helpful because it relates to gene translation and protein processing and has useful clinical correlations. Individuals with class I, II and III variants usually have a more severe phenotype, whereas individuals with class IV, V and VI variants have some residual function of CFTR protein and have atypical CF manifestations. As with any classification system, there are limitations. For example, although the most common variants, p.Phe508del, is predominantly a class II trafficking variant, some protein is trafficked to the PM where it is not functional as in class III gating variants and it is rapidly degraded as in class VI variants.

Another limitation of this classification is that the majority of variants have not been assigned to a class due to the lack of functional studies [5]. However, because these classes have evolved into theratypes, it is very important to also classify rare variants, so as to treat pwCF by the most adequate strategy.

CFTR protein

The protein encoded by the *CFTR* gene contains 1,480 amino acids in a single polypeptide chain, located in the apical PM of epithelial cells. It functions as a cyclic adenosine monophosphate (cAMP) regulated Cl^- channel and, as its name implies, as a regulator of other ion channels [5].

CFTR protein has a complex multi-domain structure that consists of: two transmembrane domains (TMD1 and TMD2) that form the channel pore, two nucleotide binding domains (NBD1 and NBD2), capable of ATP binding and hydrolysis (only NBD2), and a regulatory domain (RD). The correct assembly of these individual domains into a stable, yet flexible structure facilitates conformational changes, driven by phosphorylation of the RD regulated by cAMP, and ATP-binding and hydrolysis at the NBDs, which gate the channel pore formed by TMDs [13,22].

During co-translational transport, the CFTR polypeptide is integrated in the ER membrane and is N-glycosylated (core-glycosylated or immature form) of 150 kDalton.

With the aid of chaperone molecules, becomes folded (and thus, protease resistant) being then transported to the Golgi complex. In this compartment, the glycosylation groups are further modified (fully-glycosylated form) to originate a mature protein of 170 kilodalton. It is this last form that will be transported to the apical PM where it functions as Cl^- channel. Once present in the PM, CFTR undergoes cycles of endocytosis and recycling back to the cell surface. This recycling process is regulated by cAMP such that an increase in cAMP results in a net increase in the amount of CFTR proteins present in the PM. The mature protein has a half-life of > 16 h and, when damaged, it is ultimately recruited from the PM to be targeted to the lysosomes for degradation [23].

Originally, the folding process in the ER was described as very inefficient: only 25% of the produced wild-type (wt) CFTR were reported to attain the mature form, the remainder (not able to fold) being degraded in an ubiquitin-proteasome pathway (UPP) [23]. However, more recent studies have shown that this process is cell-type specific and much more efficient in the epithelial cells that normally express CFTR [24].

CFTR protein function

Epithelial tissues are composed of one or more layers of closely assembled cells that cover a surface or that line a cavity. The PM in contact with the external environment is called the mucosal or apical PM, whereas the membrane facing toward the interstitium is the basolateral or serosal PM. Both membranes have distinct roles due to their localization and different expression of proteins [25].

CFTR functions as a channel responsible for active Cl^- transport across the apical membrane which creates the driving force for sodium (Na^+) movement across the epithelium. The increased salt concentration on the luminal surface generates an osmotic driving force for water to be secreted, producing an isotonic secretion [26,25].

CFTR also exerts an inhibitory effect on the epithelial Na^+ channel (ENaC) downregulating Na^+ reabsorption [5]. In the absence of CFTR, cAMP increases the absorptive activity of ENaC by increasing its open probability. When CFTR is present, however, stimulation by cAMP results in a decrease in open probability of the ENaC channel, hereby decreasing the absorption of Na^+ ions across the epithelium [23].

CFTR expresses different functions depending on the distinct tissues it is present. In sweat glands, CFTR drives the reabsorption of salt, while in airway, pancreatic and intestinal epithelia CFTR controls the secretion of Cl^- and bicarbonate (HCO_3^-) [25].

CFTR also conduct HCO_3^- and transport of this anion will also contribute to transepithelial fluid secretion. Under normal physiological electrochemical gradients, and because CFTR is about five times more conductive for Cl^- than HCO_3^-, transport of Cl^- by CFTR accounts for the majority of fluid secretion in most secretory tissues. However, the secretion of HCO_3^- does play a key role in modulating the potential of hydrogen (pH) of the secreted fluid [25].

In addition to the CFTR function as a Cl^- channel, it also has an effect on other ion transporters including Cl^-, Na^+ and potassium (K^+) channels and the Cl^-/HCO_3^- exchanger [13].

It is important to acknowledge that in the epithelial cell and in the context of a tissue the role of CFTR is more than just that of the isolated ion channel. It is a hub in a community effort of the social network involving complex cell pathways and tissue physiologies whose connections in different tissue environments become crucial to understand from genomic, proteomic, and functional perspectives. In fact, it is the interaction of each *CFTR* variant with its proteostasis network that causes deviation from physiological pathways that causes the diseased state [27].

The importance of CFTR in the different tissues is highlighted by the consequences of CFTR malfunction in CF [28]. Environmental factors are also known to impact pwCF and there is evidence that non-genetic factors explain about half of the variation in CF lung function [29]. Described factors include second-hand smoke exposure and active smoking and ambient air pollution and outdoor and indoor allergens [29,30]. Climatology also seems to play a role, with higher temperatures and humidity increasing the risk of some bacteria lung colonization [30]. Socio-economic status often measured as a combination of education, income and occupation, can determine an individual variability in disease progression and outcome [29,30]. Lower socio-economic status influence compliance to therapies and healthcare access and are associated to worse outcomes. Lack of healthcare access include time to travel and distance to care sites, availability of specific services

within a geographic area, and financial coverage for healthcare services, all of which can be influenced by geography particularly in the Unites States of America (USA) where this has been widely reported [29,30].

Diagnosis

A diagnosis of CF is initially evoked with clinical recognition of characteristic signs and symptoms. Most of new CF diagnoses occur in asymptomatic or minimally symptomatic infants following a positive NBS result [31]. Universal NBS is only available in Portugal since 2013, thus individuals born prior to this date have not been screened [11].

CF has been included in most NBS programmes based on the well-recognized, long-term benefits of an early diagnosis. Indeed, the early determination of the presence of maldigestion enables the individual who is PI to be correctly treated with PERT preventing growth restriction and nutritional deficiencies [32]. Early identification of pulmonary infections and education of care givers about mucus airway clearance has been correlated to long-term improved lung function [33]. Delayed diagnosis of a CF child whilst symptomatic may result in long-term adverse psychosocial effects, which affect the parent–infant bond, and lead to less confidence in medical caregivers. Parents whose child tested positive for CF early via NBS experience less parental stress than parents of individuals who were clinically diagnosed [32]. Finally, a systematic literature review of mortality reported a lower CF-related mortality risk in screened cohorts [34].

Some concerns were raised regarding high levels of parental emotional stress during their wait for further diagnostic testing and when there is a false positive NBS. Also, screening approaches that include CFTR variant analysis, identify healthy infants carrying only one *CFTR* variant. From the child's perspective, the knowledge of being a carrier is not of direct and immediate benefit. Nevertheless, the benefits of NBS are now generally accepted and outcome these disadvantages [35].

Starting in New Zealand and Australia, it is now included in all US states and in most European countries. Ultimately, all NBS programmes refer infants with a positive NBS test to a specialized CF centre for sweat test and clinical evaluation so as to confirm a diagnosis of CF [11]. In Portugal, similarly to other European countries, NBS is based in immunoreactive trypsinogen (IRT) quantification in dried blood spot. Samples with IRT values above 65 ng/mL are selected for pancreatitis associated protein (PAP) determination [11]. Elevated PAP above 1.6 mg/ml confirms a positive NBS.

In individuals suspected of having CF because of a positive NBS result, the appearance of CF typical symptoms or recognition of immediate family history of CF should perform a quantitative Cl^- concentration sweat test according to approved and established protocols [31]. Usually, one sweat tests with Cl^- concentration above 60 mmol/L confirms the diagnosis of CF.

Individuals who meet sweat test criteria for CF should undergo *CFTR* genetic testing [31]. In some countries, CFTR genotype is already part of the NBS[31]. The latest classifications used in the CFTR2 project (www.cftr2.org) should be used to aid with the diagnosis. Variants are divided into four groups: CF-causing variant (individuals with two copies in separate alleles will likely have CF); Variants of varying clinical consequences -MVCC (a variant that in combination with a CF-causing variant or another MCVV may result in CF); Non-CF-causing variant (individuals with one or more are unlikely to have CF); and uncharacterized variant (it has not been evaluated by CFTR2 and may be disease causing, MCVV or benign). The identification of two CF-causing variants is consistent with the diagnosis of CF [31].

In Portugal, in a first level approach, the p.Phe508del gene variant is tested. If the result is not homozygous for this variant, genetic study proceeds using a variant kit (screening for 50 Europe-frequent gene variants) and a CF Iberian Panel kit (screening for 12 Iberian Peninsula-frequent gene variants). In the cases of one or no CF variants identified, the complete sequencing of the *CFTR* gene by next-generation sequencing is suggested [11].

If sweat Cl^- is inferior to 30 mmol/L, this indicates that CF is unlikely. On the other hand, a sweat Cl^- in the intermediate range (30-59 mmol/L) on two separate occasions will need

further study to establish or rule out a CF diagnosis. In this case, *CFTR* gene analysis and/or CFTR functional tests [10] are needed.

The passage of charged ions across cell surfaces leads to measurable electrical currents that can be measured in the nose and in the intestine using electrophysiological studies[4]. To obtain evidence of CFTR dysfunction, transepithelial nasal potential difference (NPD) measurements or intestinal current/voltage measurements (IC/VM) in native colonic epithelia *ex vivo* should be performed in a validated reference centre with trained staff [31].

Frequently, the diagnosis of CF is not easy to determine. Some individuals may have a positive NBS without clinical features consistent with a diagnosis of CF and a negative or inconclusive sweat test. In this case, if genetic testing identifies only one CF-causing variant or two variants and at least one has unclear phenotypic consequences, individuals are defined with the term CFTR-related metabolic syndrome (CRMS), in the USA, or CF screen positive inconclusive diagnosis CFSPID, in other countries, including Europe. Both these terms are now considered to be the same [31]. Prognosis and best practice for frequency and duration of follow-up are not determined for these individuals [31].

Some individuals present a monosymptomatic clinical entity associated with CFTR dysfunction that does not fulfil the diagnostic criteria for CF. This includes recurrent pancreatitis, bronchiectasis or CBAVD [31].

CFTR protein dysfunction: clinical features and management

Although lung disease is the most common cause of morbidity and mortality, CF is a systemic disease involving multiple organ systems that largely parallels the distribution of CFTR expression throughout the body (Figure 2). The concept of increased mucus viscosity and inspissation of secretions encompasses many of the manifestations of CFTR dysfunction in end organs besides the lung, including, pancreas, sweat glands, intestines, liver, sinuses and *vas deferens*.

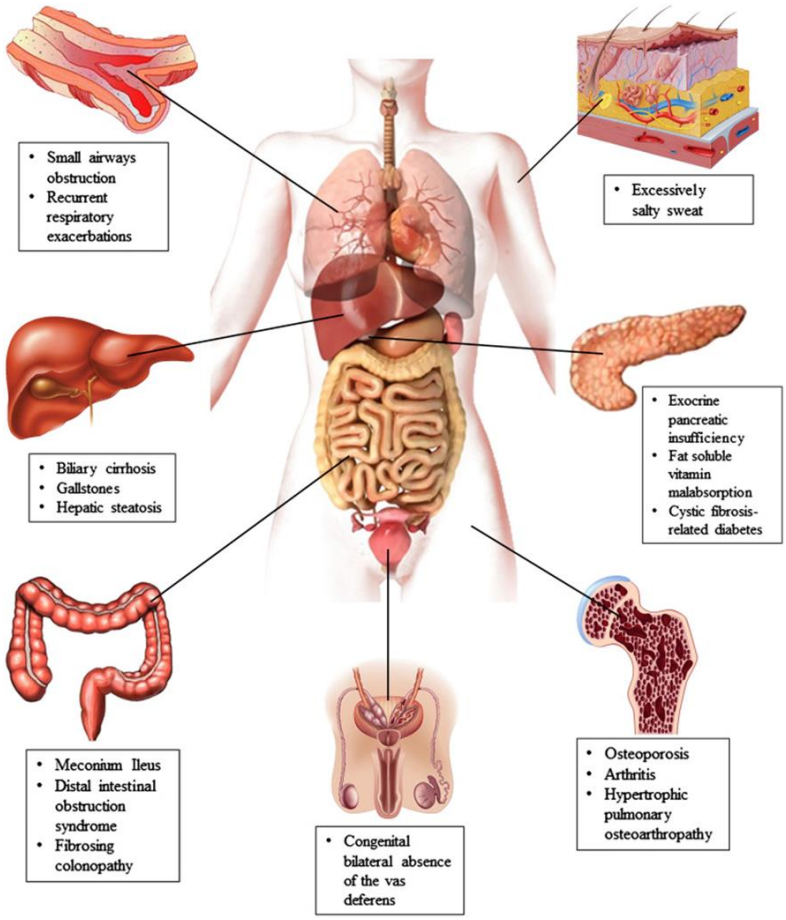


Figure 1.2. Cystic fibrosis clinical manifestations.
Retrieved from Lung Epithelial Biology in the Pathogenesis of Pulmonary Disease, Samuel A. Molina, William R. Hunt, Chapter 12 - Cystic Fibrosis: An Overview of the Past, Present, and the Future, Pages 219-249 (2017) with permission from Elsevier [36].

Sweat glands

CFTR protein is present in the PM of cells lining the sweat duct and is necessary for the reabsorption (opposite to the secretion found in other epithelial cells) of sweat Cl^- ions secreted by the sweat coil. This process is driven by the absorption of Na^+ ions through Na^+ channels present in the apical PM of the sweat duct. Individuals with CF secrete normal volumes of cholinergic (but not adrenergic) sweat in the sweat acinus, but, because CFTR protein is absent, or malfunctioning, the ion reabsorption process is inefficient and, as a result, up to five times higher sweat Cl^- concentrations are found [23]. This is the background for the use of sweat test in the diagnosis of CF.

Lungs

Pulmonary complications resulting from CF currently account for up to 85% of mortality of these individuals, and chronic cough is the hallmark of CF manifestations [37].

Lung inflammation is the dominant feature in CF respiratory tract due to chronic infections resulting from the difficulty to clear microorganisms and the production of a toxic chronic pro-inflammatory local microenvironment [38]. In children with CF, repeated respiratory tract infection with viruses and bacteria such as *Haemophilus influenza* and *Staphylococcus aureus species* results in direct and indirect damage from the inflammatory response to airway infection. As the disease progresses, disruption of innate immunity and increased mucus production, which damages peptides and proteins in the airway and digests the extracellular matrix leads to the appearance of bronchiectasis [39]. Bronchiectasis, an irreversible dilation and scarring of the airways, is a hallmark of CF lung disease [37]. It can occur in the absence of clear symptoms, and as monitored by computerized tomography (CT) scanning, it has been reported to occur in up to one-third of pwCF by the age of three years [2]. This highlights the importance of early diagnosis, disease monitoring, and intervention in young children with CF to prevent irreversible lung damage.

Later on, pwCF become susceptible to a range of Gram-negative bacteria, including the most common bacteria in CF lung infection - *Pseudomonas aeruginosa (Pa)*. Other Gram-negative bacteria are also found, such as *Stenotrophomonas maltophilia* or *Burkholderia cepacia complex (BCC)* [2]. These environmental bacteria are usually commensal organisms of supposedly low virulence which lack the inherent ability to cause disease in healthy hosts but act as opportunistic pathogens responsible for severe infections in people with underlying diseases [40,41]. These bacteria are frequently resistant to antibiotics and individuals usually require large spectrum antimicrobials [2]. Individuals with CF have recurrent pulmonary exacerbations characterized by intensified cough and sputum production, increased breathlessness and fatigue, reduced exercise tolerance and acute phase inflammatory response [2].

Monitoring of lung function has been based in spirometry tests. A forced expiratory manoeuvre allows measurement of Forced Expiratory Volume in One second (FEV1) and forced vital capacity (total volume of exhaled air) (FVC) [4]. FEV1 change has been defined as the main clinical outcome measure in clinical trials of CF drugs [4,42]. Even though this

is an objective endpoint, some individuals may not be able to perceive a modest change in their pulmonary function if they had a normal or mildly decreased pulmonary function at baseline[43,44]. Efficacy of treatments in pwCF should also be based in decreased frequency of pulmonary exacerbations, quality of life improvement and, for younger pwCF, growth improvement. These three variables are considered 'true' clinical end points but are used as secondary outcomes in clinical trials [45].

Effective treatment of exacerbations is important, as up to 25% of pwCF fail to restore lung function to the pre-event baseline and increased frequency of exacerbations leads to a faster decline in lung function, reduced quality of life and poor survival [46].

The standard management of CF lung disease includes daily airway clearance of retained mucus, treatment of lung infections (through either acute interventions targeting new infections or cycled inhaled antibiotics to manage chronic airway infections), use of mucolytic agents such as dornase alfa to thin DNA-laden mucus secretions, inhalation of mucus hydrators such as hypertonic saline and enhance clearance, and anti-inflammatory drugs to suppress the chronic neutrophilic airway inflammation characteristic of the CF airway. These treatments are effective but cumbersome, it was estimated that CF adults spend nearly two hours daily on routine preventive treatments (focused largely on lung disease management) when they are well. In addition, CF lung disease is punctuated with acute pulmonary exacerbations, in which increased pulmonary symptoms require extra interventions (such as oral or intravenous antibiotics, increased airway clearance, and frequently hospitalization) to restore lung function [37].

Gastrointestinal manifestations and nutrition

The gastrointestinal tract is one of the earliest systems affected in the course of CF and malnutrition and steatorrhea are frequently the first clinical manifestations of CF. They can be present from birth and, if left untreated, can lead to death in the first months of life. This was the picture before the 1950s, before pancreatic enzyme replacement became available [3].

Among all affected organs in CF, the exocrine pancreas is the most reliable phenotypic marker of CFTR protein function. Eighty-five percent of pwCF are pancreatic insufficient

(PI), the remainder retain sufficient residual function to be classified as pancreatic sufficient (PS). The loss of pancreatic function usually develops early in life. Waters *et al.* demonstrated that 63% of infants with CF are PI at NBS with nearly 30% of those who are PS at screening becoming PI over the next 36 months [47]. Genotype relates to pancreatic function, indeed for example, individuals with class IV, V and VI variants often are PS at birth, although some become PI later in life [2,48]. The exocrine pancreas has remarkable reserve capacity as fat maldigestion, with resultant steatorrhea, which only occurs when pancreatic lipase secretion falls below 1–2% of normal levels. Fat is the most sensitive macronutrient to malabsorption with gastric lipase production insufficient to compensate for loss of pancreatic function. Pancreatic protease (trypsin and chymotrypsin) secretion has not been assessed in relation to faecal nitrogen excretion but is likely at similarly low levels. Carbohydrate digestion remains intact despite near absence of pancreatic amylase secretion, reflecting both brush border enzyme activity and the ability of colonic bacteria to hydrolyse unabsorbed carbohydrate from the small intestine. Macronutrient malabsorption, if uncorrected, leads to acute and chronic malnutrition with weight loss and linear growth failure. Persistent protein loss is associated with development of hypoproteinaemia and oedema and accounts for the Kwashiorkor-like presentations of infants with CF in non-screened populations. In addition to macronutrient malnutrition, micronutrient malnutrition can also occur, most particularly with fat soluble vitamins. Mild–moderate steatorrhea may not alter stool appearance and growth may also be normal, as individuals can compensate by increasing food intake. Prolonged untreated PI is associated with a worse long-term outcome [47].

Measurement of faecal elastase-1 is a non-invasive simple test shown to be a reliable measure of pancreatic function[49]. Faecal elastase-1 is highly specific for the pancreas and there is no requirement to discontinue pancreatic enzyme replacement therapy (PERT) for the test[49]. Since faecal elastase-1 is measured as a concentration per gram of stool, results may be falsely low in the presence of diarrhoea. However, specificity and negative predictive value is high, so it is possible to exclude PI with 80% certainty. Faecal elastase has been suggested as a useful screening tool for longitudinal follow-up of PS individuals [47,50].

In order to counteract this maldigestion and malabsorption of macro and micronutrients and sustain normal growth, PERT is used. PERT is based on the dosage of lipase in the supplement and is dosed at 2,000-4,000 U lipase/per gram dietary fat, to a maximum of

10,000 units lipase/ kg body weight per day [50]. Careful adjustment of PERT dose with matched energy intake is important in the dietary management.

Meconium ileus is the earliest manifestation of CF, present in up to 20% of newborn infants with CF [51]. This complete obstruction of the ileum by inspissated intraluminal meconium is characterized by abdominal distension, emesis and failure to pass meconium within 48 hours after birth[49]. It is usually seen in PI newborns with severe phenotype associated *CFTR* variants [49]. Uncomplicated meconium ileus can be managed conservatively via disimpaction with enemas and intravenous hydration[51]. Complicated meconium ileus (in 40%) with peritonitis, volvulus and intestinal atresia require surgery, often with small bowel resection and primary anastomosis [48,49].

Distal intestinal obstruction syndrome (DIOS) is specific to CF [49]. It has the same pathophysiology as meconium ileus but in older individuals (more frequently in adults), characterized by the accumulation of viscid faecal material in the terminal ileum [49,52]. When complete, it may present with abdominal pain and distension, bilious vomiting, and fluid levels on radiography. Frequently a right lower quadrant mass is palpable. Risk factors include PI, dehydration, high temperatures, and history of meconium ileus and it usually treated conservatively with oral laxatives, mucolytics and enemas [49,52].

Constipation is very common among pwCF affecting nearly half of paediatric and the majority of adult individuals. Treatment is with oral laxative agents [48].

Gastroesophageal reflux disease is reported in 25-100% of pwCF [49]. Factors suggested to play a role include increased abdominal pressure due to chronic coughing, postural drainage positions during chest physiotherapy, high fat diet and prolonged gastric emptying [48,49].

Fibrosing colonopathy, with submucosal fibrosis but an intact epithelium border, was associated with excessive PERT. Since recommended maximum dosage of PERT was agreed to be 10,000 U lipase /kg per day, cases have virtually disappeared [49].

Rectal prolapse is reported in approximately 20% of pwCF. It is more frequent among young children and associated to steatorrhea before adequate PERT starts. With earlier

diagnosis because of NBS, its frequency tends to decrease. Anyway, a sweat test is recommended in any child presenting rectal prolapse [49].

There is increasing evidence to support the presence of chronic inflammation to be an important feature of the CF intestine. However, our understanding of the underlying complex pathophysiology is still incomplete [48] and a specific chapter regarding intestinal inflammation in pwCF is dedicated in this thesis (Chapter 3).

It is now recognized that pwCF have a six- to seven-fold increased risk of gastrointestinal cancers vs the non-CF population, including oesophageal, gastric, hepatobiliary, small intestinal, and colorectal cancers, particularly after transplantation [48,49].

Nutrition

Undernutrition in CF results from a combination of conditions: energy losses, high energy needs and inadequate nutrient intake. The primary cause of energy loss is malabsorption, often resulting from maldigestion of key nutrients. This can be exacerbated by low pancreatic HCO_3^- output that by not alkalinizing the intestinal lumen will impair digestive enzymes activity. Fat digestion can be further compromised if bile production is altered in the presence of concurrent liver disease. Energy losses are worsened when digestive abnormalities are associated with metabolic changes such as intestinal inflammation, small intestinal bacterial overgrowth or impaired insulin secretion [50].

Energy needs are higher in pwCF due to PI, persistent lung inflammation and infection. Individuals with CF are often unable to consume sufficient energy to overcome shortfalls. Psychosocial issues, such as anxiety and depression, chronic cough and discomfort related to other gastrointestinal problems along with the side effect of medications decrease appetite and interfere with intake goals [50].

Undernutrition affects respiratory muscle function, decreases exercise tolerance, and leads to immunological impairment [50]. In infants and young children with CF, poor nutritional status results in stunted growth, impaired cognitive function, worse lung function and poorer survival [50].

For infants and children with CF, nutritional status is considered adequate when growth is similar to that of an age-matched non-CF population: body mass index (BMI) at the 50th percentile of healthy same age population [50]. For adult pwCF it is recommended that

women maintain a BMI at or above 22 and that men maintain a BMI at or above 23 [50,53]. In the European CF registry about half of the pwCF did not achieve the adequate nutritional status goal; in the US registry nearly 25% of children were below the 10th BMI percentile and 22% of adults were underweight [50].

Along with PERT, a high-calorie, high-protein, high-fat diet is recommended. The caloric target is between 110% and 200% of the recommended daily allowance, but individual estimation of energy requirements is mandatory and should take the particular clinical situation and previous growth pattern into account [48,50].

PI increases the risk of fat-soluble vitamin (A, D, E and K) deficiency. There is international agreement that supplementation should be implemented from the time of diagnosis as deficiency states are related to deterioration of bone health (Vitamin D and K), immune function (Vitamin A and D), and inflammation (Vitamin E) [48,50].

Excessive salt losses in sweat can result in inadequate plasmatic levels of Na^+. Routine supplementation in infants is recommended. In older children and adults a Western diet should provide adequate Na^+, however supplementation is needed for exercise, hot weather or fever situations [50].

CF-related liver disease (CFLD)

There is a wide range of CFLD manifestations including: hepatic steatosis (25–70%); focal biliary cirrhosis (20–30%); micro gallbladder, sludge (15–30%); neonatal cholestasis (< 10%); multinodular biliary cirrhosis (5–10%); cholangiocarcinoma (rare) [47]. More commonly, pwCF have asymptomatic variably abnormal liver function tests. According to the European definition of CFLD, at least two of the following conditions must be present after exclusion of other causes of steatosis: hepatomegaly, liver enzyme abnormalities in at least at three consecutive determinations for 12 months, or evidence of liver disease or portal hypertension by ultrasonography [54,55].

The major determinant of clinical outcome and the pathognomonic hepatic lesion in CF is focal biliary cirrhosis, which can progress to multinodular biliary cirrhosis. Portal hypertension, which develops in about 10% of individuals with CFLD, is the main clinical

concern, often far in advance of synthetic dysfunction. While no clear genotype–phenotype correlation has been identified, CFLD seems to be confined to pwCF who are PI with severe phenotype associated variants (classes I–III) [47,54].

Ursodeoxycholic acid is widely used, and it has been shown to improve serum liver biochemistry and histological changes. It is safe but there have been no long-term studies performed, and current data demonstrate no effect on survival [47]. A Cochrane review concluded there was no good evidence to support its use [56]. The small proportion that develop cirrhosis and portal hypertension may be considered for liver transplantation. However, prior to consideration for liver transplantation, alternative "bridging" interventions have been considered by clinicians in the field including splenectomy (complete or partial) and transjugular intrahepatic portosystemic shunt (TIPS) [47].

CF-related bone disease (CFBD)

Inadequate accrual of bone mass originates in childhood. Cross-sectional studies suggest the prevalence of low bone mineral density (BMD) to be close to 50% in children; osteopenia is reported in up to 85% of adults with advanced CF disease. The evolution of low BMD in pwCF is multifactorial. CF leads to an imbalance between bone formation and absorption by disrupting the complex interplay of caloric intake, vitamins (D and K) and micronutrient (calcium, Ca^{2+} and phosphate HPO_3^-) availability, physical activity and pubertal development [48]. Glucocorticoid therapy is a strong risk factor for decreased bone mass [50].

Routine monitoring of bone health using dual energy X-ray absorptiometry (DEXA) scan from eight to ten years of age [50].

In order to prevent osteopenia individuals should be encouraged to adopt high caloric intake along with physical exercise, adequate Ca^{2+} intake and vitamin D and K supplementation. If osteoporosis develops bisphosphonates therapy may be recommended [50].

CF related diabetes (CFRD)

In CF, the endocrine pancreas is also affected over time, particularly the insulin-producing β-cells [50]. Thus, many individuals with CF eventually develop CF-related diabetes (CFRD) due to insulin deficiency. The prevalence of CFRD increases with age, so more than half of all pwCF over 40 years are affected [50]. Nevertheless, annual

screening for glucose intolerance is recommended in pwCF older than ten years of age. Treatment for CFRD includes education on diabetes self-management, insulin therapy and aerobic exercise [50].

Infertility

Ninety five percent of males with CF are infertile. Infertility results from obstructive azoospermia due to structural abnormalities of the reproductive tract, namely CBAVD [57].

Women with CF have a normal reproductive tract, however female infertility is also found in pwCF but in a much lower frequency than male infertility. In this case, it associated with undernutrition, anorexia and chronic inflammation that leads to secondary amenorrhoea [57].

Rhino-sinusitis

Nearly all pwCF will develop chronic rhino-sinusitis presenting the characteristic viscous mucus, impaired mucociliary clearance (MCC) and chronic inflammation/infection of the sino-nasal cavity. While some individuals with CF can appear relatively asymptomatic in terms of their sinus disease, commonly reported symptoms include anosmia, headache, facial pain, nasal obstruction, chronic congestion, and nasal discharge. Nasal endoscopy typically reveals mucosal oedema, purulent discharge and nasal polyposis [58]. Current treatment for CF sinusitis includes the use of hypertonic saline, topical and systemic steroids, antibiotics and endoscopic surgery [58].

Hypochloraemic metabolic alkalosis

Individuals with CF are prone to develop dehydration with salt loss and metabolic alkalosis usually occurring in regions with hot climate, due to salt wasting by sweating, and are timely recognized in pwCF. Rarely, metabolic alkalosis with hyponatremia, hypochloraemia and hypokalaemia can be the initial manifestation of CF [59].

Psychosocial effects

The effects of having a life-limiting chronic disease, progressively increasing symptoms including exacerbations, and a high burden of care can have substantial psychosocial effects on the wellbeing of affected individuals and their families. This consequently, affects adherence to treatment and hospital attendance and is associated with poor quality of life measures. High prevalence of depression and anxiety have been reported and

interventions to prevent and treat psychological distress should be available to all pwCF and their families [2].

End-stage disease in CF

The progressive effects of infection and inflammation of airways inevitably lead to a decline in lung function, frequent exacerbations and ultimately respiratory failure [60,61]. Lung transplantation has a 60-70% survival rate at five years [62] and a 45% ten-year survival rate [63]. Careful timing is key to achieving the best long-term outcomes for any individual presenting for consideration of lung transplant. Referral criteria for lung transplantation includes: FEV1 30% or less or individuals with rapidly declining FEV1 despite optimal therapy; six-minute walk inferior to 400 meters; pulmonary hypertension in the absence of a hypoxic exacerbation; clinical decline with increasing frequency of exacerbations in association with any of the following: acute respiratory failure requiring non-invasive ventilation, increasing antibiotic resistance and poor recovery from exacerbation, worsening nutritional status despite supplementation, pneumothorax and life threatening haemoptysis despite bronchial artery embolization [63].

Individuals with CF and family members should be involved in decision making regarding palliative care and palliative management should be done by the CF team that has built up a long standing relationship with the individual [60].

Innovative Therapies

Until recently (2012), the only available treatments were directed to control symptoms, but they failed to change the course of the disease. New drugs have been developed in the last decade with the potential to change the expression, function, and stability of CFTR protein, targeting the basic molecular defect: these are the CFTR modulator drugs. An update on the state-of-art regarding CFTR modulator drugs, with a special focus on the most promising clinical trials that have been carried out to date is given in detail in Part II of this chapter.

Individual-based models for Cystic Fibrosis

The concept of personalized medicine is that medical care can be tailored to the genomic and molecular profile of the individual [64]. Some define personalized medicine as referring to the use of a laboratory test to predict drug response, thereby clarifying whether or not an individual will benefit from that drug [65]. This approach is also called "Theranostics", i.e., a proposed process of diagnostic therapy for individual patients [66].

Every individual with CF is unique and the combined knowledge of gene variants along with a functional assessment of responses are crucial for assessing CFTR residual function and help to determine diagnosis (in inconclusive cases), phenotype and prognosis. For this, two validated methods are available: i) nasal potential difference (NPD); and ii) intestinal current/voltage measurements (IC/VM).

Nasal potential difference measurements

NPD is a functional examination that allows the *in vivo* measurement of voltage potential resulting from transepithelial Na^+ and Cl^- transport in secretory nasal epithelial cells[31,67]. It has been used as a diagnostic test for CF since the late 1980´s and it serves in clinical practice as a diagnostic aid in difficult cases where abnormal CFTR function is suspected[31,67,68].

The premise behind NPD measurements is that the bioelectric abnormality of the CF nasal airway, an accessible examination site, reflects transport abnormalities observed in the lower airways of pwCF [67]. Because respiratory epithelia form a tight monolayer harbouring a stable transepithelial resistance, the active secretion or absorption of charged salts such as Na^+ and Cl^- ions induces a potential difference, measured as a voltage across the epithelial surface[67]. The bioelectric potential can be measured by using a high-impedance voltmeter between two electrodes: one on the airway surface (the exploring electrode) that rests against the surface of the target epithelium and another one (the reference electrode) that can theoretically be placed in any interstitial compartment of the body, although generally the subcutaneous tissue of the forearm is used. Due to the importance of appropriate placement within the nasal cavity, and the need for an electrically quiet environment (including of the individual being assessed), some training and experience are required to achieve accuracy and reproducibility with the method [67], which is why it is not available in many countries including Portugal.

Intestinal current/voltage measurements

CFTR is the dominant apical Cl^- channel in the intestine[69,70]. It is highly expressed in intestinal epithelia, offering high specificity and sensitivity for the testing of CFTR function[69]. Ion transport in the intestine is a very sensitive measure of CFTR function: it is estimated that only 10% of wt-CFTR is necessary to prevent intestinal pathology in CF, and a very small gain in *CFTR* expression (from 1% to 5% of wt) results in large gains in Cl^- secretion (from 5% to 25% of wt levels). Because of this sensitivity, IC/VM is used to better characterize variants of unknown disease liability or to confirm a diagnosis of CF in the context of intermediate sweat Cl^- levels [71].

A freshly obtained rectal biopsy is mounted into small aperture open-circuit micro-Ussing chambers continuously perfused with a buffer solution[69]. After equilibration, basal bioelectric properties are recorded, and the effects of pharmacological stimuli on intestinal ion transport can be studied *ex vivo* in a controlled setting. Intermittent current pulses are applied to estimate transepithelial resistance (R_{te}), this allows the direct measurement of the charge flux across the tissue at a given clamp voltage thus reporting the ion transport capacity (current) of the epithelium. The biopsy material is then exposed to a sequence of drugs: (1) amiloride: to block Na^+ absorption through the ENaC; (2) carbachol (CCH): cholinergic co-activation of basolateral K^+ channels, that increase electrical driving force for luminal Cl^- exit via CFTR; (3) indomethacin: inhibits endogenous cAMP formation via the prostaglandin pathway; 3-isobutyl-1-methylxantine (IBMX) / forskolin (Fsk): to activate cAMP dependent Cl^- secretion. The evaluation relies primarily on the cAMP-mediated Cl^- secretion as measured by the change in the transepithelial current (ICM) or voltage (V_{te}) difference (IVM). In IVM, the respective change in the equivalent sort-circuit current (I_{sc}) can be deduced from this value using Ohm's law ($I_{sc}=V_{te}/R_{te}$) [70,72,73].

Application of CCH under basal conditions elicits lumen-positive responses in CF and lumen negative in non-CF-tissues (Figure 1.3 A, B, C). Nevertheless, due to variable levels of endogenous prostaglandins, lumen-positive responses can also be observed in non-CF control tissues, in the absence of a prostaglandin pathway inhibitor (e.g., indomethacin). Thus, when CCH is applied for a second time, now under indomethacin to completely inhibit endogenous cAMP (and thus CFTR-mediated Cl^- secretion), all tissues present lumen-positive responses correspondent to K^+ exiting in the cell (Figure 1.3 A, B, C)[74] . Next, when IBMX and Fsk is used to activate cAMP dependent CFTR-

mediated Cl^- secretion (IBMX/Fsk), lumen-negative responses are observed in tissues from individuals who are either non-CF (Figure 1.3A) or from pwCF with residual function (Figure 1.3B) but lumen-positive responses for those in with classic CF sub-group (Figure 1.3C) [74]. Finally, following stimulation with CCH in the presence of IBMX/Fsk, three different response patterns are observed and quantified in relation to pre-reagent (IBMX/Fsk) baseline values, namely:

i. monophasic lumen-negative (Cl^-–secretory) in tissues from non-CF controls (Figure 3 A);

ii. monophasic lumen-positive (K^+-secretory) in tissues with Classic CF (Figure 3C);

iii. biphasic responses, in the CF tissues with residual CFTR function (Figure 3B).

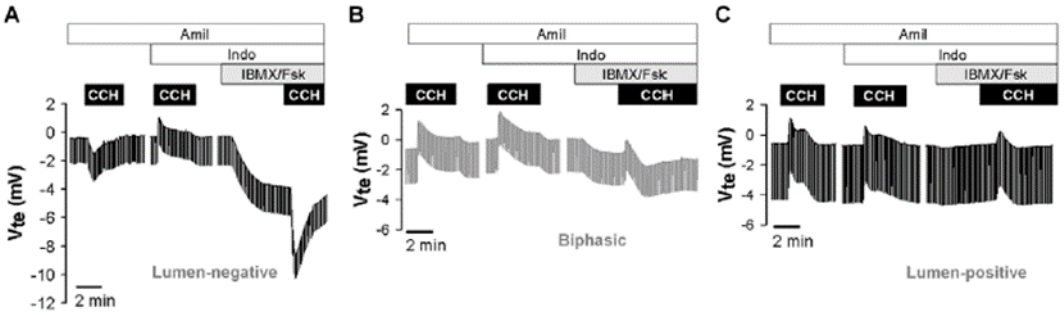


Figure 1.3. Exemplification of results from Ussing chamber measurements in rectal biopsies.
Effects of cholinergic (CCH) and cAMP-dependent (IBMX/Fsk) activation on transepithelial voltage in rectal tissues from (A) healthy control presenting CCH lumen-negative response; (B) tissue from a pwCF evidencing residual CFTR function, as shown by th biphasic response and (C) tissue from an individual with classic CF, i.e., with no detectable Cl- secretion, presenting only lumen-positive responses. All the experiments were performed in the presence of amiloride (Amil, luminal) and indomethacin (Indo, basolateral). Reproduced with permission from PLoS One (2012) [74].

Compared to airway tissues, rectal biopsies can be easily obtained by minimally invasive procedures and rectal tissue has several advantages for studies of CFTR function. First, rectal tissues express higher levels of CFTR than airway tissue. Second, in contrast to the airways, the rectal epithelium expresses alternative Ca^{2+}-activated Cl^- channels at low levels and, therefore, both cAMP and Ca^{2+}-mediated Cl^- secretion are mostly related to CFTR function. Finally, the intestine including the rectum is not affected by chronic

infection with CF pathogens or structural organ damage and remodelling, factors that may impede CFTR Cl^- channel function independent of the basic molecular defect of CFTR variants[69,70].

Human cellular models

Despite the success of the novel drugs there are still 10-15% of pwCF without any CFTR targeted treatment. Only five CF causing variants in the European Cystic Fibrosis Society (ECFS) Patient Registry have an allele prevalence above 1%. Thus, most variants are rare and affect a small number or even only one person worldwide, making classic drug development programs difficult and reinforcing the importance of personalized medicine [16].

The alternative to conventional clinical trials lies in the use of models based on individuals own tissue to predict responses to CFTR modulator drugs at an individual level.

Tools to assess the effectiveness of drugs in an individual – derived model are required, so as to predict the best combination of medicines for each candidate. This defines the term "theranostics" that was brought to the CF field (see above) [16].

Several preclinical model systems can potentially be used to predict the response of an individual to a given compound. There are heterologous cells lines expressing mutant *CFTR* gene (e.g. Fischer rat thyroid -FRT- cells) and patient-derived models. The later have the advantages of taking into account each individual's response (theranostics) globally, i.e., including the effect of modifier genes [16].

Patient-derived models systems include primary human bronchial airway epithelial cells (HBE), human nasal epithelial (HNE) cells and intestinal organoids [1,7,75].

Well differentiated primary HBE cell cultures are considered the gold standard to validate pathophysiological pathways in CF research and have proven very successful for drug development as a preclinical validation of CFTR modulator efficacy. Most HBE cells are obtained from explanted lungs in large quantity, however obtaining them through bronchoscopy is invasive and the number of cells obtained for culture is limited [16]. Drug efficacy tests performed in explanted lungs will no longer benefit the individual him or herself, the main goal of personalized medicine.

HNE cells, freshly obtained via nasal brushing have recently become an alternative on which to measure CFTR-mediated Cl^- secretion. It can be measured in monolayers of HNE cells, similarly to studies in HBEs. However, specific expertise and time are necessary to achieve successful HNE expansion and optimal and standardized culture conditions need to be established [16].

Intestinal organoid cultures are three-dimensional primary stem cell cultures that self-organize into tissue-recapitulating ”mini-guts” *in vitro* that enable the long-term expansion of primary patient rectal biopsies using defined growth conditions (Figure 1.4) [7].

CFTR protein, when activated by cAMP stimuli, plays a major role in rapid fluid secretory responses in the large intestine. CFTR protein malfunction dysregulates epithelial Cl^- and fluid transport, resulting in secretory diarrhoea.

In rectal organoids, CFTR is expressed on the apical membrane that lines the internal lumen. CFTR activation by forskolin, a cAMP-raising agent, leads to a Cl^- transport into the organoid lumen that is accompanied by luminal water secretion through osmosis. A functional assay has been developed to quantify CFTR function in intestinal organoids that rely on the luminal Cl^- secretion and water transport – the forskolin-induced swelling (FIS) assay [76].

Incubation of organoids with forskolin leads to rapid luminal fluid secretion through CFTR activation that causes whole organoid swelling in 60 min. FIS results are full CFTR-dependent as demonstrated by the absence of FIS in organoids lacking functional CFTR or when CFTR function is inhibited by chemical CFTR inhibitors, [76]. Incubation of organoids with different drugs may lead to different FIS results indicating that some drugs rescue CFTR function. By studying the effects of drugs on different genotypes, the optimal CFTR modulating drug for each genotype can be identified [1].

Intestinal organoids have demonstrated a robust ability to predict drug efficacy in individuals with CF [7,77]. Intestinal organoids are relatively easy to cultivate and expand, although they still require expertise and time; they have high *CFTR* gene expression levels; they can be biobanked as living cellular systems with infinite capacity and high reproducibility; they can later be thawed and expanded again for testing responsiveness

to new *CFTR* gene modulators coming out of the pipeline [78]. The main readout to quantify the CFTR function and how it can be rescued by CFTR modulators is the FIS assay. Forskolin raises intracellular cAMP that leads to opening of the CFTR ion channel and subsequent ion and fluid transport ("swelling") into the organoid lumen in a CFTR-dependent manner [79]. In this way, FIS assay can be used to quantify the function of the CFTR protein in response to CFTR modulating drugs.

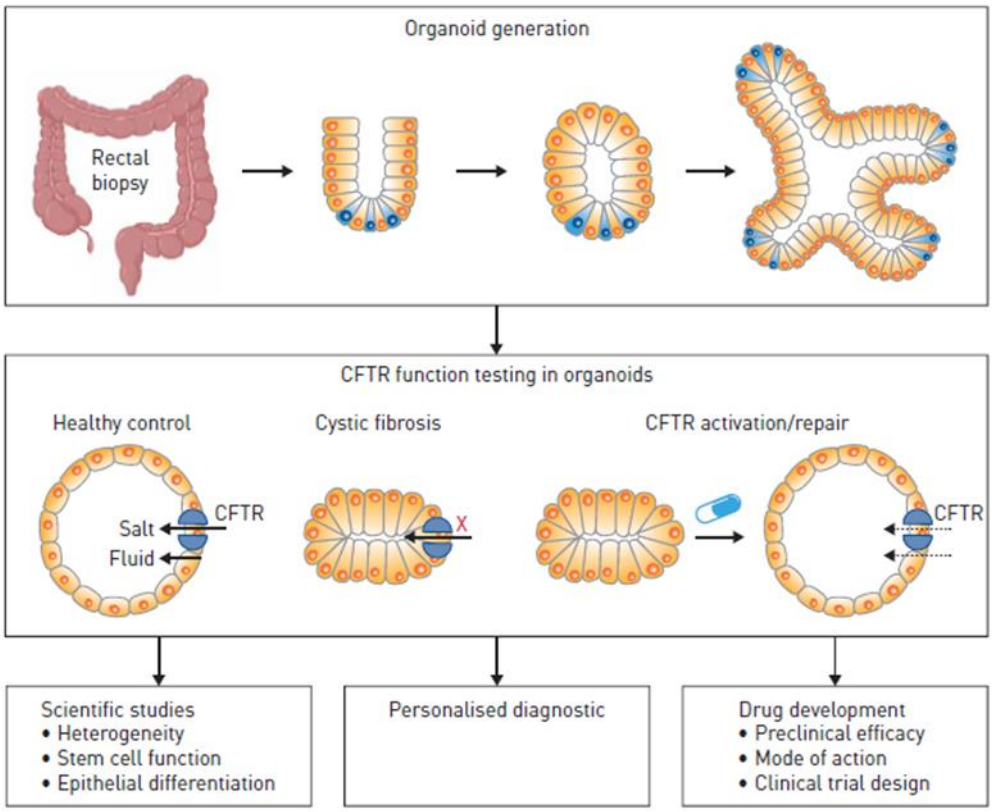


Figure 1.4. Potential applications of intestinal organoids to study Cystic Fibrosis.
Intestinal organoids can be generated from rectal biopsies. In organoids, robust CFTR function measurements can be performed based on phenotypic differences between organoids and the observation that repair and activation of CFTR causes swelling of organoids. This model can be used in preclinical and clinical studies with the potential to obtain individual-specific information on disease severity and CFTR-modulator drug response. Reproduced with permission of the ©ERS 2022 [76].

Results from intestinal organoids have been correlated with IC/VM and sweat Cl^- concentration in the same individual [1,80]. A correlation between organoid responses and changes in sweat Cl^- concentration and lung function improvement *in vivo* has been shown across individuals with different *CFTR* gene variants and with use of different CFTR modulators [7,79,81].

The relationship between results in individual-derived materials and long-term outcomes remains to be established and these data will only be gathered as time progresses. In

individuals with common variants such as p.Phe508del there is marked inter-individual variability in FIS response to CFTR modulators which is consistent to the individual's phenotype variability. To what degree these variations among individuals with the same genotype also correspond to differences in lung or pancreatic function or sweat Cl^- still needs to be established [16].

Aims

The main objective of this doctoral work is to characterize clinically and genetically a small paediatric cohort of individuals with CF for a potential personalized therapeutic intervention with CFTR modulators using intestinal organoids.

Secondary objectives:

1. **Clinical and genetic characterization of a cohort of 23 individuals with Cystic Fibrosis from the centre region of Portugal**

My first goal is to clinically and genetically characterize 23 children with CF from the centre region of Portugal diagnosed and followed in the Cystic Fibrosis Reference Centre of Hospital Pediátrico of Centro Hospitalar e Universitário de Coimbra (HP-CHUC) so as to identify potential candidates to administer new CFTR modulators.

Particular interest will be deposited in gastrointestinal manifestations of CF and relating faecal inflammatory markers to histologic inflammatory findings.

2. **Assessment of CFTR function in each individual's own tissue**

To assess CFTR function in native colonic tissue from each pwCF, using intestinal voltage measurements (IVM) in order to determine CFTR dependent Cl^- secretion, and correlate these functional data with both clinical data and the respective genotype.

3. **Drug response assessment**

To analyse the response to currently approved CFTR modulator drugs *ex vivo* in native colonic cells - intestinal organoids - from individuals followed at our centre, in order to predict which individuals are likely to respond to these drugs.

Part II - New drugs in Cystic Fibrosis: What has changed in the Last Decade?

This chapter contents are included in the paper: Roda J, Pinto-Silva C, Silva IAL, Maia C, Almeida S, Ferreira R, Oliveira G. New drugs in cystic fibrosis: what has changed in the last decade? Ther Adv Chronic Dis. 2022 May 21;13:20406223221098136. doi: 10.1177/20406223221098136. PMID: 35620188; PMCID: PMC9128052.

Abstract

Cystic fibrosis, a life-limiting chronic disease caused by variants in the cystic fibrosis transmembrane regulator (CFTR) gene, affects more than 90,000 people worldwide. Until recently, the only available treatments were directed to symptom control, but they failed to change the course of the disease. New drugs developed in the last decade have the potential to change the expression, function, and stability of CFTR protein, targeting the basic molecular defect. The authors seek to provide an update on the new drugs, with a special focus on the most promising clinical trials that have been carried out to date. These newly approved drugs that target specific CFTR variants are mainly divided into two main groups of CFTR modulators: potentiators and correctors.

New therapies have opened the door for potentially disease-modifying, personalized treatments for pwCF.

Introduction

Cystic fibrosis is an autosomal recessive monogenic disease that affects more than 90,000 people worldwide. It constitutes the most common life-limiting genetic disease in the Caucasian population [14,82,83]. The clinical manifestations of CF are caused by a defect in the Cystic Fibrosis transmembrane regulator (CFTR) protein, a Cl^- channel that is widely distributed on epithelial surfaces [84]. CFTR plays a central role in the coordination of electrolyte and fluid transport in a variety of epithelial tissues, including airways, gastrointestinal tract, reproductive tract and secretory glands, maintaining the volume and liquidity of the luminal compartment and its contents [25,85]. Its dysfunction leads to disruption in airway clearance of mucins and increases in bacterial colonization, pancreatic insufficiency and intestinal obstruction [85].

There are many variants in CF with diverse molecular effects, contributing to the variable phenotype of the disease. Additional contributing effects are due to epigenetic factors, environmental influences, modifier genes and complex CFTR alleles [86]. Although the disease affects multiple systems in the body, the symptoms in the respiratory system by

far contribute the most to the morbidity and low life expectancy characteristic of the disease [87].

Until recently, the only available treatments for CF were symptomatic and included the use of mucolytic agents, inhaled mannitol and hypertonic saline, inhaled and systemic antibiotics and anti-inflammatory drugs [87]. In the last decade, enormous progress has been made and new drugs have been developed with the potential to change the expression, function and stability of CFTR defective proteins, the so-called CFTR modulators [83,87]. Along with NBS (available in Portugal since 2013) and early and intensive symptomatic therapy, these new drugs with possible disease-modifying effects are expected to contribute to an increase in the life expectancy and quality of life of pwCF [88]. Improvements in these approaches and new corrective strategies are currently under clinical investigation [83,87,89].

Here, we provide an overview of the new drugs that are currently approved to restore CFTR function, covering the molecular bases of their mechanism of action, and focusing on the most promising clinical trials that have been carried out to date.

Molecular basis of CFTR protein modulators

A classification system that distributes CFTR variants into seven classes has been developed and constitutes a useful tool for the development of pharmacotherapy, since similar defects may respond to similar strategies [19]. Despite the existence of a classification system, over 2,100 variants have been described, most of which have not been characterized, and several of them present pleiotropic defects [89–91]. The CFTR 2-Clinical Function and Translation of CFTR (https://cftr2.org/) is a database of CFTR variants that includes data from over 89,000 individuals from worldwide CF centres and targets to provide up-to-date summaries of genotype-phenotype information. To date, 485 variants are included in the CFTR 2 database, 401 of which are CF-causing, 49 have varying clinical consequences, 24 are non-CF causing and 11 are of unknown significance.

CFTR modulators are small molecules that aim to enhance or even restore the function of defective CFTR proteins by different approaches. There are five categories of modulators: potentiators, correctors, stabilizers, read-through agents and amplifiers that apply to different mutation classes (see Chapter 1 – Part I) [15,89]. Potentiators (e.g., Iva) act on CFTR channels that have reached the cell surface and increase the open probability (gating) and conductance of ions [92]. Correctors (e.g., Lumacaftor, Tezacaftor and Elexacaftor) act on misfolded CFTR and permit delivery to the cell surface, thereby improving the channel density at the PM [92]. Since the most prevalent variant in individuals with CF, p.Phe508del, is a class II variant associated to a folding/trafficking CFTR defect (see Chapter 1 – Part I), the importance of this type of modulators is even greater. Stabilizers (e.g. cavosonstat) decrease the endocytosis rate of CFTR proteins that are present at the PM [87]. Amplifiers (e.g., PTI-801) are expected to increase the mRNA expression of CFTR and increase protein biosynthesis, which are potentially important for all variant classes [15]. Read-through agents (e.g., ataluren) are intended to promote the ribosomal read-through of premature termination codons in CFTR mRNA [87,92]. Information regarding the different classes of CFTR variants and their prevalence, including some examples of variants of each class and directed therapeutic approaches, are shown in Table 1.1 and Figure 1.5.

Table 1.1. CFTR variant classes regarding their main defect, their prevalence, some examples and directed therapeutic approaches.

Defect	**Class I**	**Class II**	**Class III**	**Class IV**	**Class V**	**Class VI**
	Protein synthesis	**Protein traffic**	**Channel gating**	**Channel conductance**	**Protein abundance**	**Plasma membrane protein stability**
	More severe phenotype			**Milder phenotype**		
Prevalence	10%	70% (at least one allele)	4-5%	3%	3%	---
Examples	p.Gly542X p.Trp1282X p.Arg553X	p.Phe508del p.Ile507del p.Gly85Glu p.Asn1303Lys	p.Gly551Asp p.Ser549Arg p.Val520Phe	p.Arg117His p.Arg334Trp p.Ser1235Arg	p.Ala455Glu	r.p.Phe508del p.Gln1412X
Therapeutic approach	Read through agents + Stabilizers	Correctors + Potentiators + Stabilizers	Potentiators + Stabilizers	Correctors + Potentiators	Stabilizers + Amplifiers	Stabilizers + Amplifiers

CFTR, cystic fibrosis membrane regulator; r, rescued

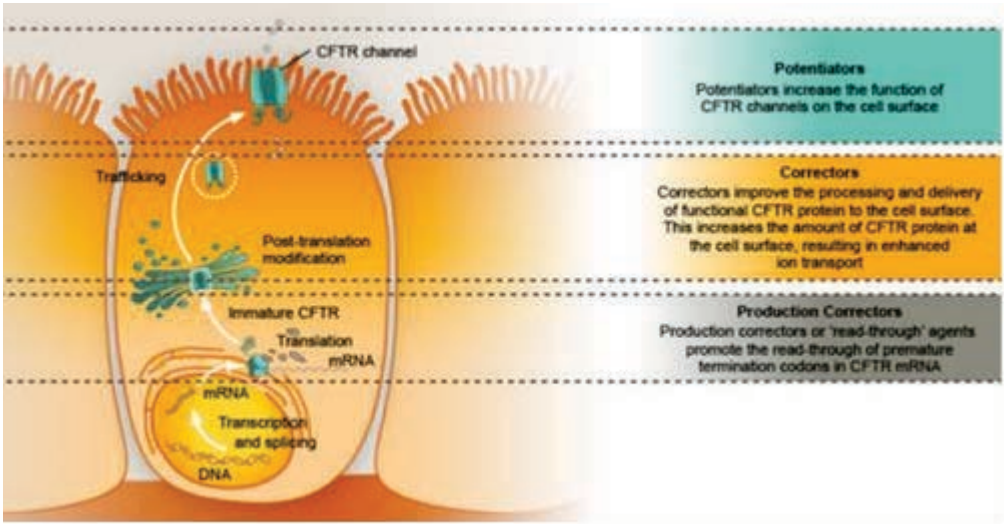


Figure 1.5. Site and mechanism of action of different CFTR modulator drugs.
Source: *Reused with permission from De Boeck* [93].

To date, four different modulators have reached clinical practice - potentiator VX-770 or Ivacaftor, abbreviated as 'Iva' (commercial name: Kalydeco®), combination therapy with a potentiator and a corrector: Lumacaftor (VX-809; 'Luma') /Iva (commercial name: Orkambi®), Tezacaftor (VX-661; 'Teza')/Iva (commercial names: Symdeko® in North-America Symkevi® in Europe) and triple therapy combining two correctors and a potentiator: Elexacaftor (VX-445, 'Elexa')/Teza/Iva (commercial names: Trikafta® in North-America, Kaftrio® in Europe).

Clinical trials of CFTR modulators

Potentiator Iva

Preclinical trials identified Iva (VX-770) as a promising substance to increase the activity of defective CFTR protein. It acts as a potentiator with the greatest effect in cells with a substitution of glycine for aspartic acid at amino acid 551 in the CFTR protein (p.Gly551Asp-CFTR variant), with a favourable pharmacokinetic profile [94]. Many clinical trials with notable results have subsequently been conducted, and Iva (Kalydeco®) was approved by the Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) in 2012. Ivacaftor was the first medicine to treat the underlying cause of CF, constituting a major breakthrough [15,83].

Initial clinical trials showed that Iva was effective in improving lung function by a greater than 10.6% change from baseline through week 24 in predicted FEV1 in the Iva group compared to placebo (P<0.001). Other outcomes were an improvement in quality of life, as well as a reduction in the number of exacerbations. Iva also proved to be effective in weight gain both for individuals with atypical lung disease and normal spirometry as well as those with severe lung disease (FEV1 < 40%). The drug was well tolerated in all clinical trials and raised no major concerns regarding safety [95–99]. Initially, the studies encompassed children older than six years of age and with the p.Gly551Asp-CFTR variant only; however, new clinical trials have expanded these indications.

Subsequent studies assessing the efficacy and safety of Iva in pwCF and a non-p.Gly551Asp gating variant (p.Gly178Arg, p.Ser549Asn, p.Ser549Arg, p.Gly551Ser, p.Gly1244Glu, p.Ser1251Asn, p.Ser1255Pro, p.Gly1349Asp) also proved that Iva was effective in monotherapy at improving lung function without raising major safety concerns for other class III variants; therefore, its use was extended [100]. For p.Arg117His, a residual function mutant, Iva also improved FEV1 in individuals older than 18 years and with a polythymidine tract variant, but all individuals showed clinical benefits, increasing Iva usage indications [101]. Currently, Iva is approved for a total of 38 CF-causing variants, comprising splicing variants and other rare variants (Table 1.3) [15]. Several studies have demonstrated the lack of clinical effects of Iva in monotherapy for individuals homozygous for the p.Phe508del variant, demonstrating the pleiotropic defects of this variant and the need for a combination of drugs with different mechanisms of action to obtain more efficient rescue of this mutated protein [102].

The KIWI clinical trial expanded the use of Iva to children older than six months (in the US), showing that Iva was generally safe and was associated with rapid and sustained reduction in sweat Cl^- concentrations (Tables 1.2 and 1.3) [103–105].

Table 1.2. Summary and simplified presentation of the absolute changes in relation to placebo of the most relevant clinical trials.

Drug	Age	Variant	Sweat Cl^- (mmol/L)	FEV1 improvement	Other significant data
Ivacaftor (KALYDECO®)					
Phase III STRIVE [95]	≥12 years	p.Gly551Asp	-48.1	10.5%	55% reduction in pulmonar exacerbations; average weight gain 2.7 kg
Phase III ENVISION [97]	≥6 years	p.Gly551Asp	-53.5	10.0%	Average weight gain 2.8 kg
Phase III KONNECTION [100]	≥6 years	Class III variants non p.Gly551Asp (p.Gly178Arg; p.Ser549Asn; p.Ser549Arg; p.Gly551Ser;p.Gly1244Glu; p.Ser1251Asn; p.Ser1255Pro or p.Gly1349Asp)	-49.2	10.7%	Average BMI increase 0.7 kg/m2
Phase III KIWI [105]	≥2 years and ≤5 years	At least one allele with class III variant	-46.9	NA	Liver enzymes increase in 15%
Phase II DISCOVER [102]	≥12 years	p.Phe508del Homozygous	-2.9	NSS	Primary and secondary outcomes not achieved
Luma					
Phase II [106]	≥18 years	p.Phe508del Homozygous	-8.21	NSS	
Luma+*Iva* (ORKAMBI®)					
Phase III TRAFFIC [42]	≥12 years	p.Phe508del Homozygous	NA	2.6%	34% pulmonary exacerbations reduction
Phase III TRANSPORT [42]	≥12 years	p.Phe508del Homozygous	NA	3.0%	43% pulmonary exacerbation reduction; BMI increase 0.36 kg/m^2
Phase III [109]	≥6 and ≤11years	p.Phe508del Homozygous	-24,8	2.4%	BMI increase +0.15 kg/m2; QoL; LCI (-0.88)
Tezacaftor + Iva (SYMKEVI® or SYMDEKO®)					
Phase III EVOLVE [110]	≥12 years	p.Phe508del Homozygous	-10.1	4.0%	35% pulmonary exacerbations reduction
Phase III EXPAND [111]	≥12 years	p.Phe508del + residual function variant	-9.5	6.8%	
Elexacaftor + Teza + Iva (KAFTRIO® or TRIKAFTA®)					
Phase III 112	≥12 years	At least one allele with p.Phe508del	-41.8	14.3%	

BMI, body mass index; FEV1, forced expiratory volume in the first second; LCI, lung clearance index; NA, not applicable/not evaluated; NSS, not statistically significant; QoL, quality of life score.

Table 1.3. Approved CFTR modulator drugs. Product names and dosages, approved individuals ages and types of CF variants eligible.

Substance	Commercial name	Variants	Approval age
Iva	Kalydeco	p.Glu56Lys, p.Gly178Arg, p.Ser549Arg, p.Gly1244Glu, P67L, p.Glu193Lys, p.Gly551Asp, , p.Ser1251Asn, p.Arg74Trp, p.Leu206Trp, p.Gly551Ser, p.Gly1069Arg, p.Ser1255Pro, p.Asp110Glu, p.Arg347His, p.Asp579Gly, p.Arg1070Gln, p.Asp1270Asn, p.Asp110His, p.Arg352Gln, p.Ser945Leu, p.Arg1070Trp, p.Gly1349Asp, p.Arg117Cys, p.Ala455Glu, p.Ser977Phe, p.Phe1074Leu, p.Arg117His, p.Ser549Asn, p.Phe1052Val, p.Asp1152His, 3272-26A->G, 711+3A->G, p.Glu831X, 3849+10kbC->T and 2789+5G->A	>12 months >6 months (US)
Luma/Iva	Orkambi	p.Phe508del homozygotes	>2 years
Teza/Iva	(US) Symdeko (EU) Symkevi	p.Phe508del homozygotes or p.Phe508del heterozygotes with p.Glu56Lys, P67L, p.Arg74Trp, p.Asp110Glu, p.Asp110His, p.Arg117Cys, p.Glu193Lys, p.Leu206Trp, 711+3A→G, p.Arg347His, p.Arg352Gln, p.Ala455Glu, p.Asp579Gly, p.Glu831X, 2789+5G→A, p.Ser945Leu, p.Ser977Phe, p.Phe1052Val, K1060T, A1067T, p.Arg1070Trp, p.Phe1074Leu, 3272-26A→G, p.Asp1152His, p.Asp1270Asn, 3849+10kbC→T	>12 years
		p.Phe508del homozygotes or p.Phe508del heterozygotes and variant with residual function as approved for > 12 years (see above)	>6 years (US)
Elexa/Teza/Iva	(US) Trikafta (EU) Kaftrio	At least one p.Phe508del variant	>12 years

CF, cystic fibrosis transmembrane regulator; EU, Europe, US, United States of America.

Corrector Lumacaftor

Lumacaftor (Luma, VX-809) is a CFTR corrector discovered through high-throughput screening. The safety, tolerability and pharmacokinetics of Luma were evaluated in a phase II, randomized, multicentre, placebo-controlled clinical trial in adults homozygous for the p.Phe508del variant [106]. This study demonstrated that modulation of CFTR function with Luma in monotherapy was safe in p.Phe508del homozygous individuals but insufficient to match clinically relevant results [106].

Combination therapy Lumacaftor (corrector) + Ivacaftor (potentiator)

A phase II, randomized, multicentre, multidose and placebo-controlled clinical trial was performed in individuals aged 18 years or older who were homozygous and heterozygous for the p.Phe508del variant [107]. The results revealed that the dose of Luma 600 mg (id) + Iva 250 mg (2id) in homozygous individuals for p.Phe508del was the only dose that

demonstrated a significant effect on FEV1, and this therapy did not have a clinically significant effect in individuals heterozygous for p.Phe508del [107].

Based on the previous results, two phase III randomized, double-blind, placebo-controlled clinical trials were conducted to assess the effects of the combination of Luma/Iva in individuals aged 12 years and older who were homozygous for the p.Phe508del variant [42]. In these clinical trials, called TRAFFIC and TRANSPORT, individuals were randomly assigned to one of the following groups: Luma 600 mg (id) + Iva 250 mg every 12 hours, Luma 400 mg every 12h + Iva 250 mg every 12h or placebo in the same regimen for 24 weeks [42]. The primary outcome was to assess the absolute change from the baseline value of FEV1. For the results, there was an average absolute increase in FEV1 in relation to placebo from 2.6 to 4% ($p < 0.001$) in TRAFFIC and 2.6 to 3% ($p < 0.001$) in TRANSPORT. This effect occurred within 15 days from the start of treatment and remained consistent for 24 weeks. The rate of pulmonary exacerbations also decreased significantly compared to placebo, and there was an improvement in BMI z score (0.24-0.28, $p < 0.001$- in the dataset) [42]. Based on data from this study, the combination of Luma/Iva was approved by the FDA in July 2015 and by the EMA in November 2015 for the treatment of pwCF homozygous for the p.Phe508del variant older than 12 years of age [18].

In the PROGRESS extension clinical trial [108] in which TRAFFIC and TRANSPORT individuals participated, it was found that both the long-term safety and benefits of the Luma/Iva combination were maintained. Subsequently, a clinical trial was carried out to evaluate the safety and efficacy of the combination of Luma/Iva in children aged six to eleven years who were homozygous for the p.Phe508del variant [109]. FEV1 did not improve significantly until week 24 (+ 2.5% over the baseline level, $p = 0.0671$), but there were improvements over the initial baseline level in sweat Cl^- concentration (-24.8 mmol/l, $p < 0.0001$), BMI z score (+0.15, $p < 0.0001$), quality of life and lung clearance index (LCI) (-0.88, $p < 0.0018$). Based on these two clinical trials, in January 2018, the EMA approved the combination of Luma/Iva for the treatment of pwCF at least six years old and homozygous for the p.Phe508del variant. This indication was extended to those who were two years of age in Europe in January 2019 [22] (Tables 1.2 and 1.3).

Combination therapy Tezacaftor (corrector) + Ivacaftor (potentiator)

Tezacaftor (Teza, VX-661) is a CFTR modulator that belongs to the corrector class, similar to Luma. The EVOLVE clinical trial aimed to evaluate the efficacy and safety of the Teza/Iva combination in subjects aged 12 years and over and homozygous for p.Phe508del [110]. The results showed an average absolute increase in predicted FEV1 over placebo of 4% ($p < 0.001$) and a 35% reduction in the rate of pulmonary exacerbations in the combination therapy group compared to placebo ($p < 0.005$). In the EXPAND extension study [111], which aimed to evaluate the efficacy and safety in subjects aged 12 years or older with one p.Phe508del *variant* and another residual CFTR function variant, the mean FEV1 increased by 6.8% ($p < 0.001$) compared to the baseline level and compared to placebo in the combination.

Based on two phase three studies, EVOLVE and EXPAND, the Teza/Iva combination was approved in February 2018 by the FDA and in October 2018 by the EMA. Thus, Teza/Iva, is approved for pwCF 12 years or older who are homozygous for the p.Phe508del variant or with a p.Phe508del variant and another variant that demonstrates residual function or responsiveness to Iva (Tables 1.2 and 1.3).

Triple therapy: elexacaftor (corrector) + tezacaftor (corrector) + ivacaftor (potentiator)

Elexacaftor (Elexa, VX-445) is the latest approved CFTR corrector, which together with Teza and Iva, has proven to be the most effective combination in the treatment of CF. A randomized, double-blind, placebo-controlled phase III clinical trial was carried out, including 403 individuals aged 12 years and over with at least one p.Phe508del variant and one variant with no function. Patients were divided into two groups: one group that received the triple combination for 24 weeks and one placebo group, and the main objective was to assess the change in the absolute value of the FEV1 percentage after four weeks [112]. In the triple therapy group, there was a 13.8% increase in the predicted FEV1 at four weeks and a 14.3% increase at 24 weeks. The rate of pulmonary exacerbations was 63% lower, and there was an improvement in the quality of life and a 41.8 mmol/L decrease in sweat Cl^- concentration. Mild to moderate adverse effects and the need to discontinue the medication due to adverse effects occurred in 1% [112]. FDA approved this triple therapy, branded as Trikafta®, in October 2019 and allowed the extension of these

modulating drugs to all individuals who have at least one allele with the p.Phe508del variant (approximately 80% of individuals with CF). EMA approval was obtained in 2020 under the trade name Kaftrio® [93] (Tables 2.2 and 2.3).

Anti-inflammatory effects of CFTR modulators

Iva lowered the sputum levels of neutrophil elastase (NE), interleukin (IL) 8 and IL-1β in the first year of treatment along with reducing the concentration of *Pa* [113]. In addition, this agent enhanced the antibacterial activity of ciprofloxacin [114]. The effect of Iva was monitored via the decreasing plasma level of human epididymis protein 4 (HE4) as a new inflammation-specific biomarker in CF that showed a reverse association with improved FEV1% values [115].

In the serum samples of CF subjects, IL-18 and Tumor Necrosis Factor (TNF) -α were significantly downregulated upon treatment with both Luma/Iva and Teza/Iva, while only the latter therapy could reduce IL-1β[116]. Via the functional rescue of dysfunctional CFTR using cellular models, an anti-inflammatory effect of Luma/Iva treatment in response to *Pa* has been described in differentiated human bronchial epithelial cells from pwCF carrying the p.Phe508del variant[117]. Also, airway epithelial repair was improved in CF epithelial cell cultures in vitro[118]. Furthermore, the production of reactive oxygen species was dampened by Luma/Iva in peripheral blood mononuclear cells induced by A. fumigatus[119]. In parallel, HE4 expression with IL-6 was decreased via lowered p65 nuclear positivity of the NF-kB pathway in bronchial epithelial cells, which was in agreement with decreased HE4 plasma levels in individuals on Luma/Iva regimen[120].

Finally, Elexa/Teza/Iva recently demonstrated an inhibitory effect on the levels of six different ceramides causing proinflammatory and proapoptotic features in CF bronchial epithelial (CFBE) cells, suggesting the potential role of this triple combination in the modulation of CF hyperinflammation [121].

Discussion

Since the *in vitro* discovery of the first molecules capable of modulating the CFTR protein, we entered a new era in the treatment of CF, in which the underlying molecular defect could be rescued. Although medical advances in recent decades have provided symptomatic treatments that have increased the average life expectancy of these individuals, from a median age of death of 25 years in 1985 to 32.4 years in 2017 [8,122], the quality of life remains compromised, and the average life expectancy is still lower than that of the general population [123]. However, with novel CFTR modulators, the median predicted survival age for individuals born from 2013 to 2017 is now 44 years old [124].

From the first clinical trials, Ivacaftor monotherapy in class III and some class IV variants was found to produce significant improvements in the clinical symptoms of these individuals, with minimal adverse drug reactions, making it a safe and effective drug. However, the variants for which Iva monotherapy is effective correspond to a very small percentage (~5%) of pwCF [123]. The most common variant, p.Phe508del, results in a protein with several molecular defects that require a cocktail of modulating drugs, in which each one corrects a portion of the defect with possible synergistic effects on the others.

Two drug combinations, Luma/Iva and Teza/Iva, were the first approved CFTR modulator drug combinations to show significant results for the most common variant - p.Phe508del - but only when in homozygosity (or, in the case of Teza/Iva, in heterozygosity with a small number of variants with residual function).

The triple combination of Elexa/Teza/Iva is much more promising. It was shown to be highly efficient with consistently good outcomes and the potential to completely change the life expectancy of individuals with CF. Its widespread use in clinical practice is clearly highly anticipated.

Despite their approval by EMA, these drugs are not yet universally available to individuals whose indications are clear, either in Portugal or at the European level. Modulator therapies are expensive (approximately 260,000 Euro per individual, per year of treatment), which may raise the question of whether the cost-effectiveness is sustainable. However, an important fact to take into account when analysing the cost-

effectiveness of these drugs is that, more than improvement in lung function or nutritional status, they have the potential to stabilize the progression of the disease, particularly if administered early in life, before irreversible damage occurs.

This study reflects a comprehensive collection of published data on the molecular basis and clinical trials of new CFTR modulators. However, because it was not a systematic review or a meta-analysis, the authors recognize these as a limitation of this study.

Conclusion

If the defect associated with the p.Phe508del variant is fully overcome, all individuals who have at least one copy of this variant would be treated effectively, regardless of the variant on their second allele [16]. Overcoming the p.Phe508del defect could thus result in an effective treatment for the great majority (80%) of subjects with CF [16].

Nevertheless, despite the success of these novel drugs, 10 to 15% of individuals still lack any CFTR-targeted treatment options [16]. Additionally, only five CF-causing variants have a CFTR allele worldwide prevalence of >1%, which makes all other variants rare or ultra-rare and therefore difficult to find adequate variant-specific therapies in conventional clinical trials. The alternative lies in the use of models based on individuals' own tissues, such as intestinal organoids and/or human epithelial nasal cells, to predict responses to CFTR modulator drugs at an individual level [16,125].

CF management is rapidly changing, and a brighter future is expected for pwCF since CFTR modulator drugs entered clinical practice. During the period of this project new therapies were approved and a wider range of variants were included in the indications for CFTR modulators.

At present, four CFTR modulator drugs are commercially available in Portugal with specific genotype and age indications:

1. Iva: indicated for 6 years old or more pwCF and with weight superior to 25 kg, that have one R117H CFTR variant or one of the following class III variants:

p.Gly551Asp, p.Gly1244Glu, p.Gly1349Asp, p.Gly178Arg, p.Gly551Ser, p.Ser1251Asn, p.Ser1255Pro, p.Ser549Asn ou p.Ser549Arg.

2. Luma/Iva: indicated for 2 years old or more pwCF who are p.Phe508del homozygotes. Respiratory adverse reactions (e.g., chest discomfort, dyspnoea, bronchospasm, and respiration abnormal) were more common during initiation of lumacaftor/ivacaftor therapy then Teza/Iva. Serious respiratory events were seen more frequently in patients with severely compromised respiratory function and additional monitoring of these patients is recommended during initiation of therapy. Lumacaftor is a strong inducer of CYP3A, therefore co-administration with sensitive CYP3A substrates is not recommended. Drug interaction with CYP3A inhibitors (e.g. itraconazole) and CYP3A inducers (e.g. rifampicin) may determine dose adjustments.

3. Teza/Iva: indicated for 12 years old or more pwCF who are p.Phe508del homozygotes or p.Phe508del heterozygotes that also have one of the following variants: p.Pro67Leu, p.Arg117Cys, p.Leu206Trp, p.Arg352Gln, p.Ala455Glu, p.Asp579Gly, c.579+3A>G, p.Ser945Leu, p.Ser977Phe, p.Arg1070Trp, p.Asp1152His, c.2657+5G>A, c.3140-26A>G, e c.3717+12191C>T. This therapy has the same efficacy Luma/Iva but less limitations including, drug-to-drug interactions (see above) and pulmonary-related side effects (see above).

4. Elexa/Teza/Iva: for 12 years old or more pwCF who are p.Phe508del homozygotes or p.Phe508del heterozygotes with one minimal function variant. For triple therapy (there is a difference between clinical approval by EMA and the economic approval in Portugal by INFARMED for the drug to be financed by the National Health Service (NHS). Triple therapy was considered safe and efficient and, finally approved, in Europe in 2021 for individuals older than 12 years, with p.Phe508del variant in at least one allele. In Portugal it was covered by the NHS, until February 2022, only for individuals with p.Phe508del in one allele and a minimal/no function variant in the other [126].

Portugal has five CF reference centres: one in Coimbra (centre region), two in Lisbon (south region) and two in Oporto (north region). A transversal analyses of

pwCF in all these CF references centres must take place in order to provide data for the evaluation of implementing CFTR modulators in the NHS.

Chapter 2. Paediatric Population with Cystic Fibrosis in the Centre of Portugal: Candidates for New Therapies

This chapter contents are included in the paper: Roda J, Teixeira T, Silva IAI, Silva TR, Ferreira R, Amaral MD, Oliveira G. Pediatric population with Cystic Fibrosis in the centre of Portugal: Candidates for new therapies. J Pediatr (Rio J). 2022 Mar-Apr;98(2):212-217. doi: 10.1016/j.jped.2021.05.010. Epub 2021 Jul 9. PMID: 34252371; PMCID: PMC9432345.

Abstract

Background: Cystic fibrosis (CF) is a severe autosomal recessive disease that results from variants in a gene encoding the CF Transmembrane conductance Regulator (CFTR) protein, a Cl^- channel.

Objective: This study aims to characterize the clinical and genetic features of a cohort of paediatric pwCF in the centre of Portugal and to determine which ones are candidates for the new drugs modulating the CFTR channel.

Methods: A review of the demographic, genetic and clinical characteristics of pwCF undergoing follow-up in Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra was carried out.

Results: Twenty-three pwCF (12 male), with a median age of 12 years, were followed up. All individuals carry the p.Phe508del variant in at least one allele. Fifteen pwCF were p.Phe508del-homozygous, median BMI z-score was -0.13, all are PI and median FEV1 value was 78.1%. These pwCF are eligible for dual therapy (Luma/Teza+Iva) and for triple therapy (Teza+Iva+Elexa). pwCF with c.579+1G>T (n=2), p.Gln685ThrfsX4 (n=1) variants and a novel variant c.3321dup (n=1) have minimal function variant which also makes them eligible to triple therapy. Patients with a residual function variant: p.Arg334Trp (n= 3) and p.Pro5Leu (n=1) have a less severe phenotype, however in Europe, they have at present no available therapy.

Conclusion: Genetic and molecular characterization of pwCF poses an important step not just for CF diagnosis and prognosis which is tightly correlated with the clinical phenotype, but also for the eligibility of CFTR modulator drugs.

Keywords: variants, clinical manifestations, Ivacaftor, Tezacaftor, Lumacaftor, Elexacaftor

Introduction

The diversity and heterogeneity in the presentation of CF disease and its evolution are reflected in the existence of classic and atypical forms of CF, with different degrees of clinical severity, but most noticeably the latter with variability in organ involvement and delayed presentation [5]. These atypical features are essentially due to the different molecular defects present in the CFTR gene that correspond to different degrees of CFTR dysfunction, namely, in the epithelia of the respiratory, pancreatic, intestinal, hepatobiliary systems, male genital apparatus, and sweat glands. However, there is also phenotypic variability among pwCF within the same genotype, raising the question that other mechanisms and factors may influence the evolution of the disease, such as modifier genes and environmental elements [5].

CF is multisystemic and despite the great clinical variability in organ involvement, CF predominantly affects the airways leading to progressive lung disease (the major cause of morbidity and mortality), and the gastrointestinal tract, including PI and malnutrition [2,5]. Despite the identification of clinical manifestations of classical CF, additional tests are necessary to assess CFTR dysfunction and thus confirm a diagnosis of CF. The sweat test resulting in high values of Cl^- concentration (> 60 mEq/L) confirms a definitive diagnosis of CF [127,128]. In Portugal, as of October 2013, NBS for CF is carried out, initially as a pilot study and since 2018 it is integrated in the National NBS Programme. Subsequent identification of two CF-causing variants establishes a diagnosis of CF [128].

Classic CF treatment focusses on the symptoms to prevent disease progression. However, new CFTR modulator therapies which treat the molecular defects of CFTR are now in the clinical practice, but they are not yet universally available.[93,129]

Iva, a CFTR potentiator which increases the channel open probability (gating) [93,130] is of benefit in pwCF who have class III/IV variants. These variants allows the CFTR channel to be present at the PM, but has gating or conductance defects, respectively [129,130]. Clinical trials on Iva showed an improvement of FEV1 of approximately 10% in pwCF with gating variants. Teza and Luma are both correctors, which promote correct folding of

p.Phe508del-CFTR, enabling some mutant protein to be correctly transported to the PM [93,129,130]. PwCF with two p.Phe508del variants are eligible for these drugs, as this variant interferes with normal protein folding and traffic, being marked for early destruction in the proteasome [130]. However, studies showed that treatment with a corrective drug in monotherapy is not effective, and therefore the combination of correctors with a potentiator is required. In p.Phe508del-homozygous pwCF, results from clinical trials with the Luma/Iva combination showed a modest increase of 2.6-3% in FEV1, with a 40% reduction in the frequency of exacerbations; while the Teza/Iva combination revealed an increase in FEV1 of 4% and a reduction in exacerbations of 35%.[130] In Europe, p.Phe508del-homozygous pwCF can start therapy with Lum/Iva from the age of two or Teza/Iva therapy after the age of twelve. A new triple combination: Elexa/Iva/Teza has recently (2020) been approved by EMA for treating pwCF with p.Phe508del variant in at least one allele and a minimal function CFTR variant, after the age of 12 years. Clinical trials on this new therapy achieved the best results, with an increase in FEV1 of 14.3% [93]. These treatment strategies alongside the improvement in symptomatology management are expected to contribute to the increase in the average life expectancy of pwCF, which is currently in the 50's in the US [124].

It is consequently of interest to study pwCF followed up at Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra (HP-CHUC), Portugal, so that they are classified into the different functional classes according to the variants found, establish the genotype / phenotype correlation, and select possible candidates for CFTR modulator therapies.

The aim of this study is thus to characterize clinically and genetically paediatric pwCF in central Portugal and to determine which ones are candidates for Iva, Luma/Iva, Teza/Iva or Elexa/Teza/Iva.

Methods

The present study was approved by the board of the Centro Hospitalar e Universitário de Coimbra after a favourable report by the Health Ethics Committee (Ref. CHUC-080-16).

A retrospective observational study was performed, using data obtained from the last appointment of the clinical files of pwCF followed in HP-CHUC in 2019 where the following variables are routinely registered, namely: gender, current age, age at diagnosis,

genotype, nutritional status, bone impairment, pulmonary function, microbiology, and pancreatic and hepatic function.

Frequencies and respective percentages were used to summarise count data. Results were presented individually for pwCF with rare variants. For analysis that included more individuals, results were summarised using median values and the range was used to measure dispersion.

The criteria used in the diagnosis were: clinical characteristics compatible with CF, positive sweat test and genetic study with the identification of two disease-causing variants.

The sweat Cl^- test was considered positive if a conductivity test was ≥ 85 mmol/L[128] and/or the Cl^- concentration test was ≥ 60 mmol/L. Currently, the recommended sweat test is the measurement of Cl^- concentration, however, the diagnosis of older pwCF was made before this method was available.

Variants were identified for all pwCF, most as part of the diagnostic approach at the National Health Institute Dr. Ricardo Jorge. In the last years, in a first stage, the p.Phe508del variant is tested by analysing the *Amplification-Refractory Mutation System*. If the result excludes p.Phe508del homozygosity, the genetic study will be continued using the Elucigene ® *CF-EU2v1* kit (that assesses for the 50 most frequent variants in Europe) and the Elucigene ® *CF Iberian Panel* kit (assessing for the 12 most frequent variants in the Iberian Peninsula). In cases of positive Cl^- concentration test and only one or no *CFTR* variant identified, complete sequencing of the *CFTR* gene by next generation sequencing is done [11].

Nutritional status was assessed according to the z-score of weight, height, and BMI, using the following classification: i) severe malnutrition z-score BMI <-3, ii) moderate malnutrition z-score -3 to - 2, iii) normal z-score from -2 to 2 and, iv) obesity z-score ≥2.[131,132] Bone density in the lumbar spine was assessed using DEXA osteodensitometry (dual-energy X-ray absorptiometry). The first bone density assessment was done between eight and ten years of age and results from the last DEXA scan were considered. if osteodensitometry Z-score is below -2 it is considered to be significantly decreased [127].

Lung function was defined based on the FEV1, considering a value of ≥80% as normal. Spirometry tests are only applied to pwCF from the age of six [127,133]. Chronic airway infection refers to individuals in whom airway samples were culture positive for the same bacterium in more than 50% of the samples obtained over the last 12 months (minimum 4 samples per year) [134]. Exocrine pancreatic function was assessed using faecal elastase levels considering mild exocrine insufficiency, i.e., PS (between 100 and 200 µg / g) and severe, i.e., PI (<100 µg / g) [128]. Liver disease associated with CF is defined as the presence of at least two of the following: hepatomegaly, changes in transaminases or ultrasound-suggested anomalies [128].

Results

Twenty-three people from the centre of Portugal with a diagnosis of CF were followed up at the HP-CHUC, and their CFTR genotypes grouped according to the functional classes. Twelve were male and 11 female. Median age was 12 years (range: 2 months - 18 years).

The initial manifestation that led to the CF diagnosis was: a positive NBS test (n=7, 30%); respiratory symptoms and failure to thrive (n=4, 17%); respiratory symptoms only (n=4, 17%); failure to thrive only (n=1, 4%); meconium ileus (n=5, 22%); nasal polyps (n=1, 4%); and hyponatraemic and hypochloraemic dehydration (n=1, 4%).

The age of onset of the disease clinical manifestations varied from the first days of life to 15 years, with a median of 3 months (Table 2.1).

Table 2.1. Demographic and clinical characterization of pwCF according to functional classes of their genotypes.

	Classe II	Class IV			Class VII				unknown
Genotype	p.Phe508del/ p.Phe508del n=15	p.Phe508del/ p.Arg334Trp n=3			p.Phe508del/ c.579+1G>T n=2		p.Phe508del/ p.Gln685ThrfsX4 n=1	p.Phe508del /c.3321dup n=1	p.Phe508del / p.Pro5Leu n=1
Gender	7F: 8M	M	F	F	M	F	F	M	M
Age (years)	8 (2m-17y)	17	14	9	15	12	17	2	2
Meconium ileus	3	no	no	no	yes	no	no	yes	no
Neonatal screening[a]	7 positive	-	-	-	-	-	-	negative	positive
Age at diagnosis	2 m (2m-5y)	15y	13y	9m	1m	1 m	2y	3 m	1 m
Sweat test[b] **(mmol/L)**	112 (med)	112	102	91	108	108	119	120	87
Pancreatic function	15 PI	PS	PS	PS	PI	PI	PI	PI	PS
BMI z-scores	-0,13 (-4,7 – 1,4)	-0,4	1,2	0,9	-0,7	-2	-4,9	-0,2	-0,3
CFRLD	2 yes	no	no	no	yes	yes	yes	no	no
Bone density[d] **(z-score)**	-1,7	-1,6	0,6	[d]	-0,3	-2,3	-4,6	[d]	[d]
FEV1 % [e]	78 (34-110)	85	90	109	107	30	25	[c]	[c]

N, normal; PS, pancreatic sufficient; PI, pancreatic insufficient; y, years; m, months; M, male; F, female; BMI, body Mass Index; CFRLD, CF related liver disease; FEV1, forced expiratory volume in 1 second.

[a]Only available after 2013

[b]Sweat conductivity test

[c]Pulmonary function test is only performed in children older than 6 years old

[d]Lumbar DEXA scan. Usually, performed in children older than 8-10 years old (Eight individuals with nine years were excluded)

[e]Eight individuals with < 6 years were excluded

Currently, 20% of pwCF have severe or moderate malnutrition and their median BMI z-score is +0,25; 83% have exocrine PI, 16% have low bone mineral density for their chronological age (Table 2.1), 36% have lung function impairment and median FEV1 is 85% and 52% have chronic infection with one or more bacteria.

The genetic study identified six different variants and, as expected, p.Phe508del was the most frequent one (allele frequency: 80%) present in homozygosity in 15 pwCF (60%).

Analysing the data by functional classes, we found that the 15 pwCF with the homozygous p.Phe508del variant (Class II) have a median age of eight years (2 months - 17 years); the median age at diagnosis is two months (ranging from 1 month to 5 years); they have a median BMI z-score of -0.13 (ranging from -4.17 to +1.42); all these pwCF suffer from PI. The median sweat test value was 112 mmol/L (range: 78 mmol/L to 124 mmol/L). The median FEV1 value was 78% (range: 34% to 110%). Three of these pwCF

(20%) had chronic lung infection with a bacterium, the most frequent being *Staphylococcus aureus*. Two have liver disease and two have low bone density (Table 2.1).

The heterozygous pwCF all have the p.Phe508del variant in one allele and in the other, variants were: Class IV: p.Arg334Trp (legacy name R334W) (n = 3); Class VII: c.579+1G>T (legacy name 711+1G->T) (n = 2) and p.Gln685ThrfsX4 (legacy name 214insA) (n = 1); not classified: p.Pro5Leu (legacy name P5L) (n = 1). A new variant - c.3321dup - was also not yet classified but it leads to frameshift and therefore could be considered Class VII (n = 1). Functional studies to assess CFTR are in progress to confirm the classification of this variant.

Individuals with class VII variants are 2 to 18 years old, the age at diagnosis ranges from 1 month to 2 years old and all are PI. One of these individuals has severe malnutrition and another one has moderate malnutrition. The median sweat test value was 114 mmol/L (range: 108-120 mmol/L). The median FEV1 value was 68% (range: 25-107%). In this group, all individuals have chronic infection with a bacterium, the most frequent ones being *Pa* and *Bcc*. Three have liver disease and two suffer from low bone density. The new variant was found in a 2-year-old male toddler with a clinical history highly suggestive of CF (meconium ileus, PI, chronic lung infections, and respiratory failure) and, although his NBS test was negative, he had two positive sweat Cl^- tests.

Individuals with class IV variants are represented here by three pwCF who are 9, 14 and 17 years old. Two of these pwCF were diagnosed as adolescents. They are all PS, they have an adequate nutritional status, and their pulmonary function is normal. Two pwCF have chronic lung infection, one with *Pa* and the other one with *Staphylococcus aureus*.

The child with the still unclassified p.Phe5Leu variant is 2 years old, had an adequate nutritional status, PS and with no respiratory symptoms.

Two homozygous pwCF for the p.Phe508del variant, were already in combination therapy Luma/Iva. One 16-year-old girl had a severely compromised lung function (40% predicted FEV1 and chronic lung infection with *Bcc*), PI and moderate undernutrition when treatment was started. After two years in treatment, her nutritional status improved and is now adequate and lung function stabilized. Another 13-year-old girl who has been

on Luma/Iva therapy for almost one year, remained with severely compromised lung function (FEV1 34%) but her nutritional status slightly improved being now adequate.

Discussion

The aim of this study was to clinically and genetically characterize paediatric pwCF in central Portugal and to determine which ones are potential candidates for new CFTR modulator drugs. We found that analysing clinical data and grouping individuals into functional classes according to their genotype gives us an idea of the severity and prognosis of the disease in each pwCF. It also helps to identify which individuals may benefit from available CFTR modulator drugs for which eligibility is variant-specific.

We are aware of the limitations of our study as it is based on a small number of pwCF from only one centre. Particularly, conclusions when comparing characteristics from different functional classes are limited because of the small number of pwCF in each class.

Comparing the current group of pwCF under follow-up at HP-CHUC with the paediatric population described by the US CFF 2018 annual report, there are no significant differences in terms of nutritional status (median BMI z-score -0.2 vs +0,2, respectively). Lung function is slightly decreased in the pwCF at our centre but still in the normal range (FEV1 82% vs 97%, respectively) [124]. The most frequent initial manifestations did not deviate from the classically referred in the literature, but the diagnosis was more challenging in some less common initial manifestations, such as nasal polyps and hyponatraemic hypochloraemic dehydration [135].

The frequency of the p.Phe508del variant was equal to the 80% reported for Portugal, as well as for Northern and Central European countries [8].

There was great phenotypic variability among pwCF with the same genotype, particularly in pwCF homozygous for p.Phe508del or with class VII variants, which appears to be independent of the age/duration of the disease. In the same group, there were pwCF with malnutrition and impaired lung function and others with adequate nutritional status and normal pulmonary function, which suggests that genetic and/or environmental factors

also influence the disease severity. Further studies are underway in order to analyse other factors, like socio-economic status and compliance to therapy [27].

Interestingly, the individual with the new class VII variant and meconium ileus as initial manifestation, had a false negative NBS test. This fact, which is described in the literature, is justified by the earlier and severe destruction of the pancreas during gestation and the consequent lower concentration of IRT and PAP at birth [11,93].

We found that the three pwCF with Class IV variants seem to have a less severe phenotype. This may be explained by the residual function associated with CFTR protein present in individuals with these variants.[2] Previous studies have also described significantly lower sweat Cl^- values in pwCF with class IV variants in comparison to pwCF homozygous for p.Phe508del and a later age at diagnosis [136].

Since the approval of Luma/Iva and Teza/Iva drugs for pwCF homozygous for p.Phe508del, 15 pwCF followed-up at our centre would benefit from it. However, among these pwCF only two individuals are currently on Luma/Iva therapy. Also in the US CFF 2018 report about 20% of pwCF considered eligible for treatment with CFTR modulators are not taking it essentially because it is very expensive [124]. In fact, these drugs are not yet accessible to all pwCF in all European countries. Currently, great hope is being placed on the highly effective CFTR modulator therapy (HEMT)[137], it was licensed for use by pwCF aged over 12 years who have two copies of the p.Phe508del variant or one copy of p.Phe508del and one copy of a 'minimal function variant' [93]. Nineteen of our pwCF will be eligible to HEMT triple therapy, including p.Phe508del-homozygous and p.Phe508del-heterozygous with a minimal function variant (c.579+1G>T, p.Gln685ThrfsX4 and c.3321dup).

Patients with one residual function variant (Class IV): p.Arg334Trp (n= 3) and p.Pro5Leu (n=1) have an atypical phenotype, however in Europe, they have at present no available therapy. Because no pwCF carries a gating variant, Iva is not indicated for any of these individuals.

Ideally, all individuals that fulfil the approved indications should immediately benefit from treatment, particularly with HEMT which has showed the best results. However negotiations between individual country governments and the pharmaceutical industry are underway since the very high cost has been a major issue [138]. For now, centres must

decide which individuals should start these therapies first. This poses the question: should we choose pwCF who already have severe and most probably irreversible consequences of their disease, or should we choose individuals who still have normal lung, pancreatic or liver functions, i.e., before they start to deteriorate? Should we choose adults or children?

Clinical trials that led to CFTR modulators approval are based on clinical outcomes, the main one being FEV1 improvement. However, an important data to take into account when analysing the cost-effectiveness of these drugs is the fact that, although the improvements in lung function are modest, the simple fact that they are potentially able to stabilize the progression of the disease, particularly if administered early in life and before irreversible damage, can prove to be of great importance for pwCF, health professionals and families struggling to contain the inexorable clinical deterioration characteristic of CF.

In conclusion, the genetic and molecular characterization of pwCF poses an important step not just for CF diagnosis and prognosis which is tightly correlated with the clinical phenotype, but also for the eligibility of CFTR modulator drugs, namely HEMT.

Chapter 3. Faecal calprotectin and rectal histologic inflammatory markers in cystic fibrosis – a single centre study

This chapter contents are included in the paper: Roda J, Maia C, Almeida S, Oliveira RC, Ferreira R, Oliveira G. Faecal calprotectin and rectal histological inflammatory markers in cystic fibrosis: a single-centre study. BMJ Paediatr Open. 2022 Apr;6(1):e001422. doi: 10.1136/bmjpo-2022-001422. PMID: 36053631; PMCID: PMC9058793.

Abstract

Objective: To analyse the association of faecal calprotectin with the genetic and clinical characteristics of paediatric people with Cystic Fibrosis (pwCF). In a subset of these individuals, we aimed to associate histologic inflammatory features of rectal mucosa to faecal calprotectin levels.

Methods: In a prospective study, faecal calprotectin levels were collected in all 23 pwCF attending our paediatric centre, together with demographic and clinical data. Correlations between faecal calprotectin and clinical features were determined. In 11 of these individuals, endoscopic rectal biopsies were obtained and the association between faecal calprotectin and histologic inflammatory markers was analysed. Statistical analyses included Spearman´s correlation coefficient, Mann-Whitney and Exact Fisher tests. Sensitivity and specificity was calculated.

Results: Median age of pwCF was 12 years, 19 had pancreatic insufficiency (PI) (19/23). Seventeen (17/23) had elevated faecal calprotectin, and the median value was 88 µg/g (Interquartile range (IQR)=178 µg/g). Higher faecal calprotectin levels were observed in the PI group (101 *vs* 30 µg/g, P=0.027). No significant correlation between elevated faecal calprotectin level and BMI z-score was found. Five individuals (22%) reported abdominal pain, three (13%) complained of diarrhoea and three (13%) had constipation, but these symptoms were not associated with elevated faecal calprotectin.

Unspecific focal rectal inflammation was found in four individuals (4/11). An association between rectal mucosa inflammation and elevated faecal calprotectin was found (p=0.015). Sensitivity was 100% and specificity was 86%.

Conclusion: In pwCF analysed here, elevated faecal calprotectin was frequent, particularly if PI, and it was not related to gastrointestinal symptoms nor malnutrition. Elevated faecal calprotectin was present in individuals with histologic evidence of rectal inflammation. Faecal calprotectin may be an indicator of asymptomatic rectal inflammation in pwCF.

Introduction

CF is also the most common cause of PI in children [139]. Historically, CF children died in infancy from severe malnutrition and later from respiratory failure, but due to improved clinical care, including PERT, currently, a majority of them reach adulthood [140]. Approximately 85% of pwCF have impaired digestion due to PI and despite adequate PERT, several pwCF still have malabsorption, growth impairment and gastrointestinal problems, including abdominal pain, steatorrhea, and altered motility [139,140]. It has been suggested that digestive symptoms are attributable not only to PI but also to intestinal inflammation. However, the pathogenesis and nutritional implications of this finding remains unclear [140].

Studies, in both animals and humans, have reported evidence of intestinal inflammation in CF. In the mouse model of CF, the most common manifestation is intestinal obstruction resulting from inflammatory enteropathy, leading to perforation [141]. Furthermore, abnormal mucus accumulation in the intestines of murine models predisposes them to gut dysmotility, creating a niche for bacterial overgrowth and dysbiosis [142].

In humans, the presence of inflammatory biomarkers, including faecal calprotectin, eosinophil cationic protein, interleukin-1B and interleukin-8, was reported, suggesting that intestinal inflammation is a feature of CF [140,143]. Videocapsule endoscopic studies elicited mucosal ulceration, erythema and mucosal breaks in the small bowel of pwCF, particularly those with PI [139]. Calprotectin is a neutrophil secretory product, and elevated faecal levels are well correlated with colonic inflammation in inflammatory bowel disease [144–146]. Dysbiosis may be associated with intestinal inflammation as reflected by increased levels of faecal calprotectin that respond to antibiotic treatment [147]. CFTR modulator drugs may potentially improve dysbiosis and inflammation, for example Iva has been associated with a decrease in calprotectin levels[148].

However, to our knowledge, there are no studies characterizing intestinal histological inflammatory findings in pwCF and, particularly, studies analising the relationship between elevated faecal calprotectin and these histologic inflammatory findings.

The aim of the present study was to analyse, in a cohort of paediatric pwCF, the association of faecal calprotectin levels with both CFTR genotypes and clinical

characteristics, including nutritional status and gastrointestinal symptoms. In a subset of this cohort, we also aimed to characterize histological inflammatory features of rectal mucosa and relate it to faecal calprotectin levels.

Methods

This prospective study included children and adolescents aged 0 to 18 years followed in the Paediatric Unit of the Cystic Fibrosis Reference Centre of the Centro Hospitalar e Universitário de Coimbra, Portugal, in the year 2019. The criteria for the diagnosis of CF were: clinical characteristics compatible with CF, a positive sweat test and a genetic study with the identification of two CF-causing variants, according to the latest consensus [71]. All individuals willing to participate were included. Exclusion criteria included recent respiratory exacerbation/infection or ingestion of antibiotics, steroids, or nonsteroidal anti-inflammatory drugs, both in the previous 4 weeks.

In all participants, demographic data, CFTR genotype, and clinical data, including nutritional status and PERT medication, were collected at the time of the appointment where stool sample was obtained. Nutritional status was assessed according to the z-score of weight, height, and BMI. Patients were asked whether they had experienced gastrointestinal symptoms (abdominal pain, constipation, or diarrhoea) in the two weeks preceding the calprotectin measurement.

Exocrine pancreatic function was assessed using faecal elastase levels. PI was considered when the faecal elastase level was under 200 µg/g; above that, they were classified as PS.

Faecal calprotectin measurement

Stool samples were collected at home or at the CF Reference Centre. Calprotectin level in the faecal samples were measured using EliA Calprotectin® (reagents from Thermo Fisher Scientific®). Faecal calprotectin concentration was considered normal up to 50 µg/g stool, as was considered in previous studies with pwCF [139] and as it has been shown to be sensitive for detecting inflammation in children with inflammatory bowel disease [149].

Rectal biopsies

Rectal biopsies were obtained from pwCF with rare CFTR variants already enrolled in another study from our centre, who agreed to test responses to CFTR modulator drugs in intestinal organoids (data not published). As a consequence, most of these individuals had less common variants. Time between faecal calprotectin measurement and rectal biopsies was of a maximum of two days.

Rectal mucosa specimens (3-4 mm in diameter) were obtained from 11 individuals, with or without sedation (depending on individuals will or collaboration) using a colonoscope and colon forceps (Endoflex®, diameter 2.8 mm). Samples were immediately stored in formalin.

One pathologist with experience in gastrointestinal pathology from the Pathology Department, Centro Hospitalar e Universitário de Coimbra, Portugal performed the histologic analyses. Samples were blinded to the clinical information and were analysed under an optical microscope (Nikon Eclipse 50i®). Pictures were taken with a Nikon-Digital Sight DS-Fi1® digital camera.

Since the diagnosis of CF is based mainly on bioelectrical/biochemical analyses, there is no defined standard histological classification score. Therefore, the evaluation took into consideration the density of mononucleated inflammatory population, on a semi-quantitative approach: none, mild, moderate, and severe.

Statistical Analyses

Statistical analyses were performed with SPSS software (v.19; SPSS Inc., Chicago, IL, USA), and a p value < 0.05 was considered statistically significant. Descriptive statistics were presented according to the normality of the data distribution using Shapiro-Wilk test. Spearman´s correlation coefficient, Mann-Whitney and Exact Fisher tests were used between pwCF groups to evaluate correlation, differences, and associations, respectively. Sensitivity and specificity of calprotectin as a marker of rectal inflammation was calculated.

Ethics Approval

The present study was performed in accordance with the Declaration of Helsinki and approved by the board of the Centro Hospitalar e Universitário de Coimbra (Portugal) after a favourable report by the Health Ethics Committee (Ref. CHUC-080-16). Informed consent was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years.

Involvement of pwCF

Individuals with CF were not directly involved in setting the research question, the design or in the implementation of the project.

Results

Faecal calprotectin was measured in all 23 pwCF followed in our centre for one year. The median age was 12 years, aged from 2 months to 17 years old. Twelve were male. All PI individuals (19/23; 83%) were taking PERT. The p.Phe508del variant was present in all individuals in at least one allele. Fifteen (65%) individuals were p.Phe508del homozygous, and the others carried one of the following variants: p.Arg334Trp (n=3), c.579+1G>T (n=2), and p.Gln685ThrfsX4 (n=1), p.Pro5Leu (n=1) and a novel variant, c.3321dup (n=1) (Table 3.1). Only two individuals were on the CFTR modulator drug Luma/Iva (individuals 15 and 20), presenting faecal calprotectin level of 347 and 142 µg/g, respectively. The sweat test values for these individuals did not improve with the modulator drug.

Table 3.1. Genotype, BMI, gastrointestinal symptoms, faecal calprotectin, and rectal histology of pwCF

No	Genotype	BMI z-score kg/m^2	Pancreatic function	Abdominal pain	Diarrhoea	Constipation	Calprotectin µg/g	Histology
1	p.Phe508del/p.Arg334Trp	-0,42	PS	yes	no	no	19	normal
2	p.Phe508del/p.Arg334Trp	1,18	PS	no	no	no	15	normal
3	p.Phe508del/p.Arg334Trp	0,94	PS	yes	no	yes	79	normal
4	p.Phe508del/c.579+1G>T	-0,74	PI	no	no	yes	9	normal
5	p.Phe508del/c.579+1G>T	-1,99	PI	no	no	no	19	normal
6	p.Phe508del/p.Gln685ThrfsX4	-4,95	PI	yes	yes	no	45	normal
7	p.Phe508del/c.3321dup	-0,22	PI	no	no	no	341	inflammation
8	p.Phe508del/p.Pro5Leu	-0,3	PS	no	no	no	40	normal
9	p.Phe508del/p.Phe508del	-2,37	PI	no	no	no	223	inflammation
10	p.Phe508del/p.Phe508del	-2,6	PI	no	no	no	62	inflammation
11	p.Phe508del/p.Phe508del	-0,3	PI	yes	no	yes	63	inflammation
12	p.Phe508del/p.Phe508del	-0,64	PI	no	no	no	55	-
13	p.Phe508del/p.Phe508del	0,3	PI	no	no	no	90	-
14	p.Phe508del/p.Phe508del	0,1	PI	no	no	no	352	-
15	p.Phe508del/p.Phe508del	-0,27	PI	no	yes	no	347	-
16	p.Phe508del/p.Phe508del	-0,13	PI	no	no	no	116	-
17	p.Phe508del/p.Phe508del	0,1	PI	no	no	no	101	-
18	p.Phe508del/p.Phe508del	0,55	PI	yes	yes	no	104	-
19	p.Phe508del/p.Phe508del	1,42	PI	no	no	no	88	-
20	p.Phe508del/p.Phe508del	-1,16	PI	no	no	no	142	-
21	p.Phe508del/p.Phe508del	0,03	PI	no	no	no	55	-
22	p.Phe508del/p.Phe508del	1,1	PI	no	no	no	330	-
23	p.Phe508del/p.Phe508del	1,2	PI	no	no	no	289	-

BMI, body mass index; PI, pancreatic insufficiency; PS, pancreatic sufficiency

Seventeen of the 23 individuals (74%) had an elevated faecal calprotectin measurement, and the median value was 88 µg/g (IQR=178 µg/g). Most individuals (16/19) with PI had elevated faecal calprotectin (84%). The PI group had higher faecal calprotectin levels then in the PS group (101 µg/g; IQR=234 vs 30 µg/g; IQR=53; p=0.0027).

A moderate positive correlation was found between faecal calprotectin and sweat test values (from the time of diagnosis) (r=0.46; p=0.029). A weak negative correlation was

found between age and faecal calprotectin (r=-0.3; p=0.14). Also meconium ileus past history was not associated with elevated calprotectin (Table 3.2).

Table 3.2. Association between gastrointestinal symptoms and past history of meconium ileus and normal or elevated faecal calprotectin

	Normal calprotectin (n=6)		**Elevated calprotectin (n=17)**		
N=23	**Yes**	**No**	**Yes**	**No**	**p-value***
Abdominal pain	2	4	3	14	0.58
Diarrhoea	0	6	3	14	1
Constipation	2	4	1	16	1
Meconium ileus	1	5	4	13	1

*Fisher exact test.

Comparing the median BMI z-score in individuals with normal and elevated faecal calprotectin, no significant difference was found (-0.58 kg/m^2; IQR= 2.8 vs 0.1 kg/m^2; IQR=1.08; p=0.09). No significant correlation between faecal calprotectin level and BMI z-score was found (r=0.19; p=0.36).

All 15 individuals homozygous for the p.Phe508del variant had elevated faecal calprotectin. These pwCF have a statistically significant higher faecal calprotectin then p.Phe508del heterozygous individuals (110 µg/g; IQR=217 vs 40 µg/g; IQR=54, p=0.003).

Some pwCF reported digestive symptoms: five individuals (22%) reported abdominal pain, three (13%) complained of diarrhoea, and three (13%) had constipation (Table 3.2).

Gastrointestinal symptoms were not associated with elevated faecal calprotectin (Table 3.2).

Histologic features

Rectal biopsies were obtained from 11 of the 23 pwCF. Eight individuals had the rare variants p.Arg334Trp (n=3), c.579+1G>T (n=2), p.Pro5Leu (n=1), c.3321dup (n=1) and p.Gln685ThrfsX4 (n=1), and three individuals were homozygous for the p.Phe508del variant.

Unspecific mild focal inflammation was found in the three p.Phe508del homozygous individuals, all of which had elevated faecal calprotectin (Table 3.1). Inflammatory features were characterized as small lymphocyte and plasma cell infiltrates distributed in a vaguely nodular pattern between colonic crypts (Figure 3.1A, B, C and D).

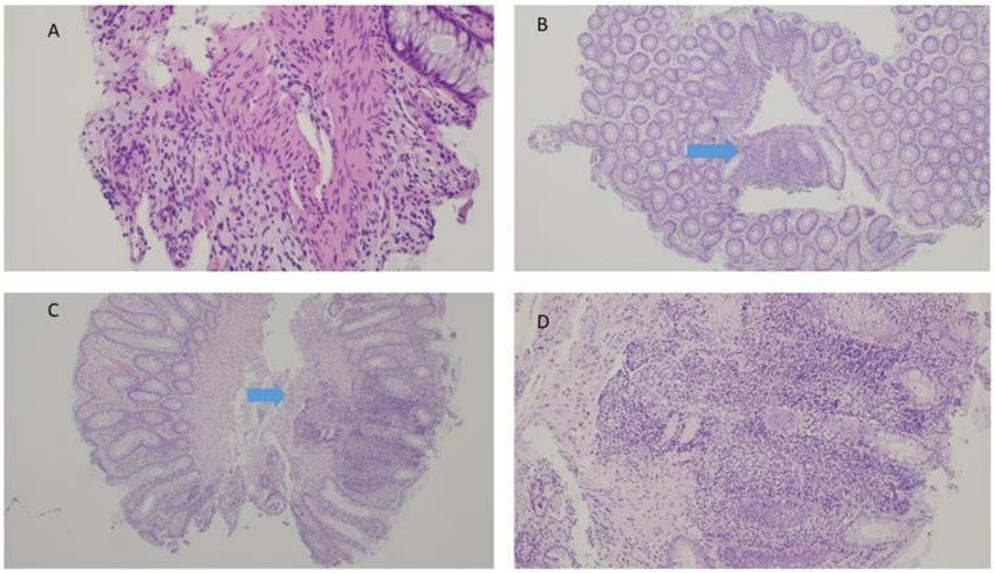


Figure 3.1. Histologic features *of:* ***A*** *individual 7 (*p.Phe508del/*c.3321dup) showing mild to moderate focal inflammation, composed of plasma cells and small lymphocytes, in the mucosa, H&E 200x;.* ***B*** *- individual 9 (*p.Phe508del/p.Phe508del*) showing mild focal inflammation of mononucleated cells of the mucosa in a vaguely nodular pattern (blue arrow), H&E 40x;* ***C*** *- individual 10 (*p.Phe508del/p.Phe508del*) showing mild focal inflammation of the mucosa, with small lymphocytes and plasma cells, between colonic crypts (blue arrow), H&E 40x, highlighted in higher magnification* ***D,*** *H&E 200x.*

Mild to moderate focal inflammation composed of plasma cells and small lymphocytes in the mucosa was found in individual 7 carrying p.Phe508del in one allele and the new variant c.3321dup in the other allele (Figure 1A). This individual had significantly elevated faecal calprotectin (341 µg/g) (Table 3.1).

There was an association between elevated faecal calprotectin levels and the presence of inflammation in rectal biopsies (Table 3.3). In our study, sensitivity of calprotectin levels was 100% and specificity was 86%.

Table 3.3. Comparison of histologic rectal inflammation presence between individuals with normal or elevated faecal calprotectin.

	Normal calprotectin n=6		**Elevated calprotectin n=5**	
N=11	**Yes**	**No**	**Yes**	**No**
Mucosal inflammation	0	6	4	1

Fisher exact test p=0.015.

Median calprotectin was higher in pwCF with histological inflammatory alterations comparing with pwCF with normal histology (143 µg/g; IQR=249 vs 19 µg/g; IQR=30; p=0.024). None of the mucosal samples had morphological changes, which are a major sign of chronic inflammation.

Discussion

Most pwCF in this study (74%) had elevated faecal calprotectin level, some reaching a maximum values > 300 µg/g. These data are consistent with previous studies and suggest the presence of intestinal inflammation in pwCF [139,140,150]. The pathophysiology of intestinal inflammation may be explained by the same triad of obstruction by mucus accumulation, inflammation and infection that causes disease in the airways of pwCF [151]. The *CFTR* gene is strongly expressed all along the intestinal tract in a cephalad-caudal gradient, *CFTR* messenger RNA levels are highest in the duodenum and levels decrease distally along the small intestine to the large intestine [151]. This "CF enteropathy" may be an independent entity in the disease process or may be due to other factors. High doses of PERT can cause inflammation and fibrosing colonopathy[152]. Inspissated intestinal secretions, mucus accumulation, constipation, slow intestinal motility, the use of proton pump inhibitors and frequent courses of antibiotics are multiple risk factors for small bowel bacterial overgrowth in pwCF, which can cause inflammation, mucosal damage and aggravate maldigestion [150]. An unfavourable intestinal microbiome may also be a stimulus for inflammation [153,154]. One trial with probiotics supported this hypothesis, as the use of *Lactobacillus rhamnosus GG* reduced calprotectin concentrations in children with CF [153]. Another study, found increased abundances of *Staphylococcus*, *Streptococcus* and *Veillonella dispar*, along with decreased abundances of Bacteroides, *Bifidobacterium adolescentis*, and *Faecalibacterium prausnitzii* to be associated to

intestinal inflammation in pwCF in similarity to changes found in individuals with Crohn´s disease [154].

In our study, lower faecal calprotectin levels were found in PS individuals. Elevated faecal calprotectin only in PI individuals has also been reported by Dhaliwal *et al* [140]. On the other hand, 16 out of 19 individuals with PI had elevated faecal calprotectin, and the difference in faecal calprotectin levels between the PI and PS groups was highly significant (101 vs 30 µg/g). This means that either PI by itself or PERT may be responsible for intestinal inflammation in these individuals[155]. However, there has been reported a lack of correlation between PERT and faecal calprotectin[156]. As reported by Dumoulin *et al.,* calprotectin is subject to proteolysis by tripsin activity, which is virtually absent in PI pwCF. As a result, calprotectin proteolysis is also reduced. Therefore, calprotectin levels detected in stools of PI individuals may be higher, and this may not be exclusively attributed to intestinal inflammation[157]. Perhaps, in PI pwCF, the upper limit of the so-considered normal faecal calprotectin should be higher than the value considered for inflammatory bowel disease, as no association was found with digestive symptoms [157,158]. Some recent studies suggest an upper limit of >50 µg/g or 250 µg/g and it remains unclear whether reference ranges that are useful in IBD are equally applicable in CF[154,158]. Also, the pancreatic status is related to CFTR function and genotype and intestinal inflammation may be another manifestation of the multisystemic involvement of the disease and not only influenced by pancreatic function [159].

Another interesting finding was that pwCF who are homozygous for p.Phe508del have significantly higher calprotectin levels in comparison to p.Phe508del-heterozygotes. This genotype may thus be associated with an increased risk of more significant intestinal inflammation.

Overall, only a small number of individuals complained of gastrointestinal symptoms, and no association between elevated calprotectin and digestive symptoms could be found. The same conclusion has been reported in studies where digestive symptoms were even much more frequent[156].

In contrast to previous studies [140], no relationship could be found between faecal calprotectin and nutritional status or growth parameters. However, interestingly, a

positive correlation was found between faecal calprotectin and sweat test values, which may be indicative of the presence of significant intestinal inflammation in individuals with a more severe phenotype. The negative correlation found between faecal calprotectin and age is in line with the reported tendency towards lower values with increasing age in healthy individuals, even though there are no well-established cut-off levels for specific age ranges[160]. However, it is in contrast with some studies that found an increase in calprotectin with age in pwCF, particularly in those with classical CF disease[161–163].

Historically, distinct histological changes, which have been interpreted as signs of mucus hypersecretion, have been reported in light microscopic studies of large intestinal mucosa from pwCF. "Hypertrophic" or enlarged goblet cells and crypts distended by accumulated mucus were described, and these changes were considered useful in the diagnosis of CF by some authors [164]. In this study we were specifically looking for histologic evidence of inflammation in pwCF and try to associate it to faecal calprotectin.

Four out of the 11 individuals in whom rectal biopsies were performed had histologic inflammatory alterations. Interestingly, all the three individuals homozygous for p.Phe508del had histological signs of inflammation, and this may be related to the classical phenotype associated to this common variant.

Elevated calprotectin levels were associated with histologic inflammation in the rectal mucosa (Table 3.3) and pwCF with rectal inflammation had significantly higher calprotectin levels. High sensitivity and specificity, allows us to conclude that faecal calprotectin may be a good indicator of rectal inflammation in pwCF. However, the clinical meaning of this finding remains to be explained, as this did not translate into more frequent gastrointestinal symptoms or influenced nutritional status. However, as the life expectancy of pwCF is increasing there has been reported an increased risk of gastrointestinal malignancies. Chronic intestinal inflammation is a risk factor for cancer development and this should probably be addressed early in life[158].

We are aware that our study had several limitations: it is a small and unicentric study, and biopsies were performed on a subset of these individuals and limited to the rectum. Larger multicentre studies with the aim of determining serial and longitudinal studies of calprotectin levels and biopsies of the upper and lower gastrointestinal tract may help to determine clinical relevance.

However, the finding of abnormal calprotectin levels and inflammatory alterations in the intestinal mucosa in the paediatric population raises questions about the early detection of CF enteropathy.

In conclusion, there is increasing evidence that intestinal inflammation is part of CF and is present early in life, particularly in childhood and adolescence. The additional contribution of low trypsin activity, chronic enzyme dosage, dysmotility, bacterial overgrowth, dysbiosis and other unidentified factors may play a role in its multifactorial cause. Fecal calprotectin may be considered a noninvasive biomarker of intestinal inflammation in pwCF since a relationship with histologic evidence of rectal mucosa inflammation was found. Further and larger studies need to be performed to confirm and explain the mechanisms and clinical relevance of these findings.

Chapter 4. Intestinal Organoids are a Good Tool to Predict Pre-Clinical Response to CFTR Modulator Drugs: a Small Portuguese Cohort

Article in preparation

Abstract

Introduction: Cystic fibrosis (CF) is the most common autossomal recessive life-shortening disease in caucasians. Therapies that rescue defective CFTR channel – CFTR modulator drugs – have reached the clinic, however these are variant-specific and accordingly, individuals with rare variants are usually excluded from clinical trials.

Aim: Our aim is to predict specific responses to approved CFTR modulator drugs by using intestinal organoids derived from rectal biopsies, from a small group of pwCF followed-up at a Portuguese CF reference centre.

Methods: CFTR-mediated Cl^- secretion was assessed in native rectal biopsies from 11 individuals with CF by Ussing chamber intestinal voltage measurements (IVM). In parallel, intestinal organoids were prepared, cultured and analysed using the Forskolin-Induced Swelling (FIS) assay by confocal microscopy [1], before and after incubation with modulators, namely: Iva, Teza/Iva or Elexa/Teza/Iva.

Results: Eight pwCF with rare variants and p.Phe508del in the other allele, were included: three with p.Arg334Trp and 1 with p.Pro5Leu (who have an atypical phenotype and in fact, exhibit CFTR residual function in both IVM and FIS assays); two with c.579+1G>T, one with p.Gln685ThrfsX4 and one with the previously undescribed variant c.3321dup (who have a classical CF phenotype and no CFTR function in IVM and FIS assay). Three pwCF were homozygous for p.Phe508del with variable clinical phenotype also participated in the study, two had no residual CFTR function (and one with no conclusive analysis).

CFTR was rescued by Teza/Iva in organoids with p.Arg334Trp and p.Pro5Leu variants. However, this combination failed to rescue CFTR function in c.579+1G>T, p.Gln685ThrfsX4, c.3321dup and p.Phe508del-homozygous organoids.

Triple therapy Elexa/Teza/Iva significantly rescued CFTR in organoids from five pwCF: two with c.579+1G>T, one with p.Arg334Trp and two p.Phe508del -homozygous.

Discussion: Our results illustrate the value of using intestinal organoids to predict individual responses to approved CFTR modulator drugs, indicating that individuals with CF bearing p.Arg334Trp or p.Pro5Leu variants may benefit from treatment with double

therapy of CFTR modulators. A more significant clinical benefit is expected for the triple therapy in pwCF carrying the p.Phe508del variant in at least one allele.

Introduction

The absence or dysfunction of the CFTR protein in the apical PM affects exocrine secretion, leading to a positive SCT, i.e., a Cl^- concentration in sweat ≥ 60 mEq/L. Disease severity correlates with organs sensitivity to CFTR dysfunction and with the amount of functional protein that ultimately is influenced by the type of variant (see Chapter 1 – Part I) [5].

Targeting specific mutant forms of CFTR with modulators has revolutionized treatment of CF, but most of these therapies are variant-specific and only apply to a specific variant, excluding individuals with rare variants (see Chapter 1 – Part II) [75].

The severity of this disease is influenced by a large number of different genes (called CF modifier genes) and biological pathways, as well as by environmental factors. Consequently, each pwCF is unique and investigational drugs should be tested *ex vivo* directly in native tissues from pwCF, towards a personalized-medicine approach with improved prognosis [5] (see Chapter 1 – Part I). Because CF is a progressive disease it is important to determine the best therapeutic option early in life before the pathological lesions become irreversible.

Intestinal organoids assays in CF rely on the basis that CFTR plays a dominant role in rapid fluid secretory responses in the large intestine that are evoked by cAMP-inducing stimuli, and its activation can lead to secretory diarrhoea. Cl^- is the main driver of this rapid fluid secretory response. CFTR is expressed on the apical PM that lines the internal lumen of rectal organoids. CFTR activation by cAMP-raising agents such as Forskolin (Fsk) leads to Cl^- transport into the organoid lumen that is accompanied by luminal water secretion through osmosis [77] (see Chapter 1 – Part I). This luminal Cl^- secretion and coupled water transport can be quantified in validated functional assays. Intestinal organoids have demonstrated a robust ability to predict drug efficacy in pwCF [7,77] and its results have been correlated with IVM and sweat Cl^- concentration in the same individual

[76]. Also, they can be biobanked, thawed and expanded again to test new and future CFTR modulator drugs coming into the pipeline [16].

Our aim here was to measure CFTR function in native tissues from pwCF, as well as to predict individual responses to approved CFTR modulators by using intestinal organoids derived from rectal biopsies of children and adolescent pwCF with rare *CFTR* gene variants.

Methods

All children and adolescents with CF followed at HP-CHUC had their genotype and clinical manifestations characterized. Patients with rare variants were all included because they are not elegible for double therapy (Luma or Teza plus Iva) and not universally elegible for triple therapy (Elexa/Teza/Iva). Patients homozygous for the p.Phe508del variant were also invited to participate in order to be used as baseline results and also to demonstrate variability among individuals with the same genotype and individualized responses to these specific drugs.

The present study follows the international ethical guidelines and was approved by the board of the Centro Hospitalar e Universitário de Coimbra after a favourable report by the Health Ethics Committee (Ref. CHUC-080-16).

Rectal biopsies

Individuals' colon preparation was performed by applying a microclister composed of gelatin 54 mg and glicerin 3830 mg or an enema of sodium dioctyl sulfosuccinate (0.01g) plus sorbitol 13.4g, 12 h prior to the procedure.

Six superficial rectal mucosa specimens (3-4 mm in diameter) were obtained from each individual, with or without sedation (depending on individuals will or collaboration), by biopsy colon forceps (Endoflex, oval forceps with diameter 2.8 mm) with visual examination[165]. Procedure was performed by the main investigator at HP-CHUC in seven pwCF and at the Pediatrics Department of Hospital de Santa Maria, Centro Hospitalar e Universitário de Lisboa Norte, Lisboa, Portugal in four pwCF.

Rectal biopsies were immediately stored in a 15ml-tube with cell culture medium (RPMI 1640) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penincilin and 1% (w/v) ceftazidime and transported in ice cold to the Amaral lab at the BioISI-Biosystems and Integrative Sciences Institute, Faculty of Sciences, University of Lisboa, Lisboa, Portugal. This is a recognized research centre where Ussing chamber intestinal voltage measurements (IVM) and intestinal organoids preparation, culture and analyses were performed by Iris Silva, Juliana Roda and Violeta Railean [74].

Intestinal Voltage Measurements (IVM)

Mounting of tissues was performed under a stereomicroscope using dissection forceps. Once the tissues were mounted on circular disk inserts (with central convex open areas of 0.0079-0.0283 cm2), these were fixed between the two-half cells of the micro-Ussing chambers, separating luminal from basolateral bath solutions (Figure 4.1).

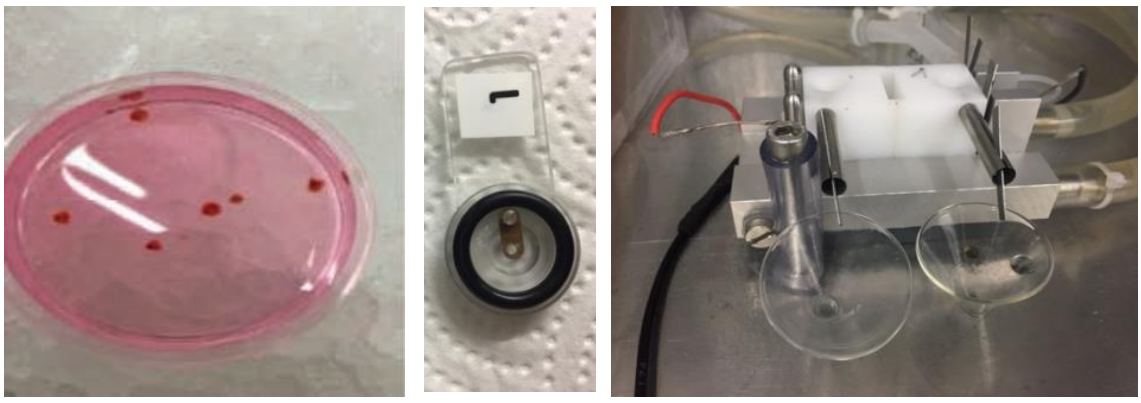


Figure 4.1. Mounting procedure of rectal biopsies onto the micro-Ussing chamber.
Rectal biopsy specimens from one pwCF (left); circular disk inserts used for rectal biopsy mounting (centre); micro-Ussing chambers arrangement for rectal biopsy analysis (right).

In these experiments, rectal biopsy specimens were mounted in modified perfused micro-Ussing chambers and analysed under open-circuit conditions at 37ºC as previously described [74,166].

The samples were continuously perfused with pre-heated (37ºC) Ringer buffer (NaCl 145 mM, KH2PO4 0.4 mM, K2HPO4•3H20 1.6 mM, D-glucose 5 mM, MgCl2•6H20 1 mM, Ca-Gluconate•1H20 1.3 mM, pH 7.4 (NaOH, HCl), 280 mOsm/Kg) followed by

experimental solutions. Values for the transepithelial voltage (V_{te}) were continuously recorded using Power Lab software (AD Instruments Inc., New Zealand) and referred to the basolateral/serosal side of the epithelium. Transepithelial electrical resistance (R_{te}) was determined by applying intermittent (1 s) current pulses (0.5 µA), measuring pulsated deviations in V_{te}. Equivalent short-circuit currents (I_{eq-sc}) were calculated according to Ohm's law ($Ie_{q-sc} = V_{te} / R_{te}$). All experimental solutions used were prepared in Ringer buffer. Amiloride (Amil, 20 µM, luminal) (Sigma-Aldrich, USA) was first applied to block electrogenic Na^+ absorption through ENaC. Next, CCH (100 µM, basolateral) (Sigma-Aldrich, USA) was added for cholinergic activation of K^+ channels and K^+ release, constituting a driving force for Cl^- exit through CFTR. Indomethacin (Indo, 10 µM, basolateral) (Sigma-Aldrich, USA) was then applied for 20 min to inhibit production of cAMP through the prostaglandin pathway and thus abolish endogenous cAMP and thus basal CFTR-mediated Cl^- secretion. CCH was then re-applied in the presence of Indomethacin as a control step for the protocol, due to general inhibition of basal CFTR activity in tissues. After the CCH washout, an IBMX/Fsk solution (100 µM/2 µM, basolateral) (both from Sigma-Aldrich, USA) was added to stimulate prostaglandin-independent cAMP production and thus stimulate cAMP-dependent (CFTR-mediated) Cl^- secretion. CCH was finally re-added on top of IBMX/Fsk for cholinergic co-activation of CFTR. Three to four rectal biopsies were measured per individual. The percentage of CFTR function in these tissues was calculated for the average maximal CFTR activation ($\Delta I_{eq-sc,IBMX/Fsk(Indo)} + \Delta I^{eq-sc,CCH(IBMX/Fsk(Indo))}$), normalized to the corresponding mean value determined previously for a reference non-CF control group (-217.45 µA/cm2) [74]. A threshold of 30% of wt-CFTR function was defined to distinguish non-CF subjects from pwCF, as described previously [72,74].

Crypt isolation and organoid culture from rectal biopsies

Three or four rectal mucosa specimens were used to isolate intestinal crypts for organoid production. The samples were washed with PBS and treated with 10 mM EDTA for 90 min at 4ºC. Crypts were isolated by centrifugation ant then cultured in 50% (w/v) matrigel, seeded at a density of 20-30 crypts in 3x10 µL matrigel droplets per well, in pre-warmed 24 well plates. The matrigel was polymerized for 15 min at 37ºC and surrounded by culture medium, as described previously[167]. Antibiotic gentamycin (Sigma 1:1000) was added to the growth medium (Primocin, 1:500; Invitrogen©) during the first

week of culture. Medium was changed every other day and organoids were passaged after 5-7 days of culturing. Detailed methods were previously described by Dekkers *et al* and Sato *et al* [80].

Forskolin induced swelling (FIS) assay

Intestinal organoids derived from the individual's rectal biopsies from a 2- or 3-week culture were seeded onto a 96-well culture plates (Thermo Fisher Scientific, Rochester, NY, USA) with 4 µL 50% Matrigel containing ~20 organoids immersed in 50 µl complete medium with or without 3µM VX-661. One day after seeding, organoids were incubated for 10 min with 3µM calcein green (Invitrogen), stimulated with Fsk with or without 3 µM VX-770 (Fsk; Fsk+VX-770; Fsk+VX-661/VX-770), and directly analysed by confocal live cell microscopy (Leica Sp8) with the 5x objective for 60 min at 37ºC. Two wells per condition were used and the experiment was repeated 3 times [7]. Later, when triple therapy became available, organoids that had been frozen in -80ºC degrees, were again seeded and incubated with Fsk+VX445/VX-661/VX-770.

The resulting swelling of organoids was quantification of live confocal microscopy images, using an in-house Cell profiler-derived software [168]. The relative increase in size of all organoids in a well was automatically calculated over time using 10-min intervals. A relative swelling curve was generated from these data and an area under the curve (AUC) of this relative swelling was calculated to compare conditions.

Without any drug, stimulation with Fsk leads to production of intracellular cAMP, which in turn activates CFTR, if it is present and functional at the PM of the organoid cells, and results in the secretion of electrolytes, followed by fluid into the lumen of the organoids, leading to their 3D-swelling. Swelling with Fsk alone (i.e., without any CFTR modulator drugs) indicates the presence of CFTR (residual) function[76].

CFTR rescue was assessed by comparison of average AUC values between control (Fsk only) and various treatments (Fsk + VX-770; Fsk + VX-661/770; Fsk+VX445/VX-661/VX-770). For [Fsk] = 0.128 µM, is established as standard for prediction of the *in vivo* treatment efficacy [7]. AUC thresholds of 750 and 1500 have been established for the prognosis of medium and high clinical benefit potential of treatments, respectively [7].

Statistical analyses

The Area Under the Curve (AUC) was calculated using Graphpad Prism version 5.01.

Statistical differences were determined with Student's t-test or analysis of variance (ANOVA), as suitable. Data are expressed as mean ± standard error of mean (SEM). Pearson coefficients (*r*) were used to find correlations and partial correlations between pwCF outcome parameters and laboratory assessed CFTR function using SPSS software (v.19; SPSS Inc., Chicago, IL, USA). P-values of <0.05 were considered statistically significant.

Results

Subjects with rare variants

All eight pwCF who carried rare variants agreed (or their parents agreed) to participate in the study. Half of these individuals (4) were females, and the median age was 13 (IQR: 12,7). The pwCF's clinical characterization is described in detail in Table 4.1.

Table 4.1. Clinical characteristics of pwCF with rare CFTR variants

	p.Phe508del/ **p.Arg334Trp Class IV**			p.Phe508del/ **c.579+1G>T Class VII**		p.Phe508del/ **p.Gln685ThrfsX4 Class VII**	p.Phe508del/ **c.3321dup Class VII**	p.Phe508del/ **p.Pro5Leu unknown**
Individual number	1	2	3	4	5	6	7	8
Gender	M	F	F	M	F	F	M	M
Age (years)	17	14	9	15	12	17	2	2
Meconium ileus	no	no	no	yes	no	no	yes	no
Neonatal screening[a]	NA	NA	NA	NA	NA	NA	negative	positive
Age at diagnosis	15y	13y	9m	1m	1 m	2y	3 m	1 m
Sweat test[b] **(mmol/L)**	112	102	91	108	108	119	120	87
Pancreatic function	PS	PS	PS	PI	PI	PI	PI	PS
Height z-score	-2.1	-0.5	1	1.01	-1.8	-2.7	-1	0.5
BMI z-scores	-0.4	1.2	0.9	-0.7	-2	-4.9	-0.2	-0.3
CFRLD	no	no	no	yes	yes	yes	no	no
CFRD	no	no	no	no	no	no	yes	no
Bone density[c]	-1.6	0,6	***	-0.3	-2.3	-4.6	***	***

FEV1[d]	85	90	109	107	30	25	**	**

BMI, body mass index; CFRD, CF related diabetes; CFRLD, CF related liver disease; F, female; FEV1, forced expiratory volume in 1 second; M, male; m, months; NA, not applicable; PI, pancreatic insufficient; PS-Pancreatic sufficient; y-years.

[a]Available after 2013

[b]Sweat conductivity test

[c]Lumbar DEXA scan. Performed in children older than eight years old.

[d]Pulmonary function test is only performed in children older than six years old

Individuals with the p.Phe508del/ p.Arg334Trp genotype

Three pwCF carried a class IV variant - p.Arg334Trp - in heterozygosity with p.Phe508del (individuals 1, 2 and 3). p.Arg334Trp (frequency of 0.00302[17]) is a CF-causing variant, also localized in TMD6 and classified as class IV due to severely reduced channel conductance but normal protein processing observed in transfected Fisher rat thyroid (FRT) cells[167,75]. Individuals carrying this variant presented variable phenotypic traits regarding pancreatic function and respiratory disease. However, normal pulmonary and pancreatic function may be present throughout childhood as described in these individuals (Table 4.1).

Individual 1 - p.Arg334Trp / p.Phe508del genotype

Individual 1 is a 17-year-old boy, whose diagnosis of CF was made at the age of 15 years, after a long course with a false diagnosis of refractory asthma and repeated pneumonias. National NBS was not available when he was born. Pulmonary function, according to spirometry tests is normal. He is PS which has contributed to adequate weight gain (BMI z-score: - 0.4). However, his stature is below the -2 z-score (Height z-score:-2.1) probably due to the delayed diagnosis.

Intestinal voltage measurements in the Ussing chamber

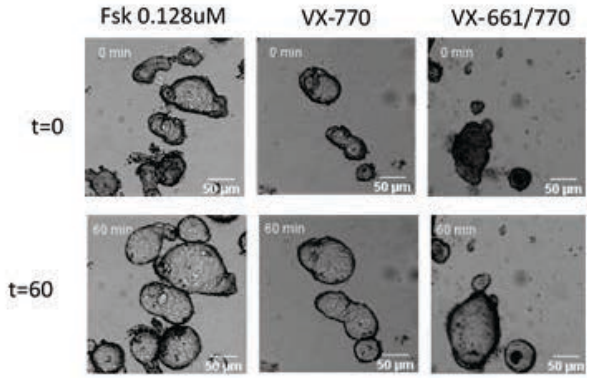
In this individual (individual 1), intestinal voltage measurements were not possible to obtain, due to poor the quality of the tissue.

Drug response

FIS results are suggestive of residual function in basal conditions, likely associated to the p.Arg334Trp allele. Modest CFTR rescue was observed with VX-770 alone and with the

combined treatment with VX-661/VX-770 at [Fsk] = 0.128 μM. These results suggest that some clinical benefit may result from Iva alone, albeit inferior to the established threshold for medium clinical benefit (red bar below the blue line in Figure 4.2B), but also indicating that potential clinical benefit for VX-661/VX-770, i.e., above the established threshold for medium clinical benefit (brown bar above the blue line in Figure 4.2B). It is thus likely that this individual will benefit from the combination VX-661/VX-770 therapy, although this drug is not currently indicated for individuals with this variant.

A



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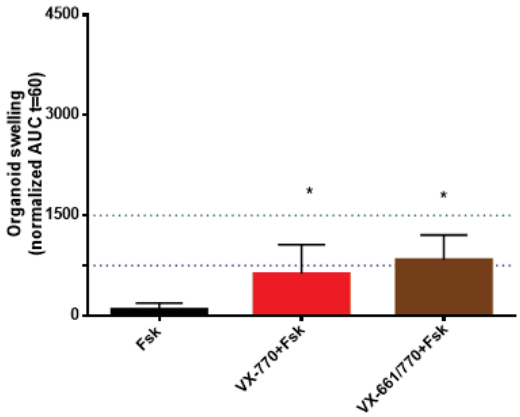
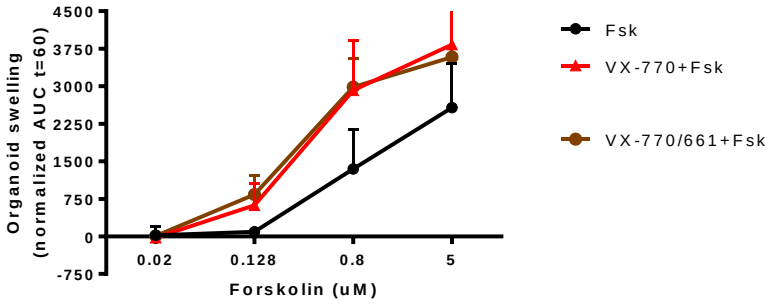




C

Figure 4.2. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 1 (p.Phe508del/p.Arg334Trp genotype).
(A) Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770 andVX-661/770, as indicated above each panel. Imagens from superior panels show organoids at the beginning of the experiment (t=0 min), before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show more swelling with VX-770 and combination therapy VX-661/770.
(B) Quantification of organoid swelling for all treatments at [Fsk] = 0.128 µM. The dashed blue and green lines represent the established thresholds for medium and high clinical benefit potential for treatments, respectively. Data represent the mean of measurements on 5-8 replicate wells per condition. (C) Quantification of FIS in organoids for all treatments tested: Fsk alone, VX-770 (3 µM) + Fsk, VX-661/770 at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 µM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min).
Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.

Individual 2 - p.Phe508del/ p.Arg334Trp genotype

Individual 2 is a 14 year-old girl, whose diagnosis of CF was made at the age of 13, subsequently to the investigation of "refractory asthma". NBS was not available when she was born. Pulmonary function, according to spirometry tests, is normal. She is PS and adequate weight gain and growth (BMI z-score is +1,2; height z-score is -0.51). No CF-related complications are present. This individual has recently started treatment with the triple therapy, and she reports less cough and tiredness and better appetite. Because FEV1 and BMI were already normal prior to treatment no objective outcome could be assessed.

Intestinal voltage measurements in the Ussing chamber

In rectal biopsies from individual 2 we found a positive response to IBMX+forskolin (IF), confirming cAMP-dependent Cl^- secretion, and Carbachol (CCH) cholinergic co-activation of K^+ channels leading to luminal Cl^- exit. This indicates the presence of partial CFTR function, i.e., residual function (Figure 4.3).

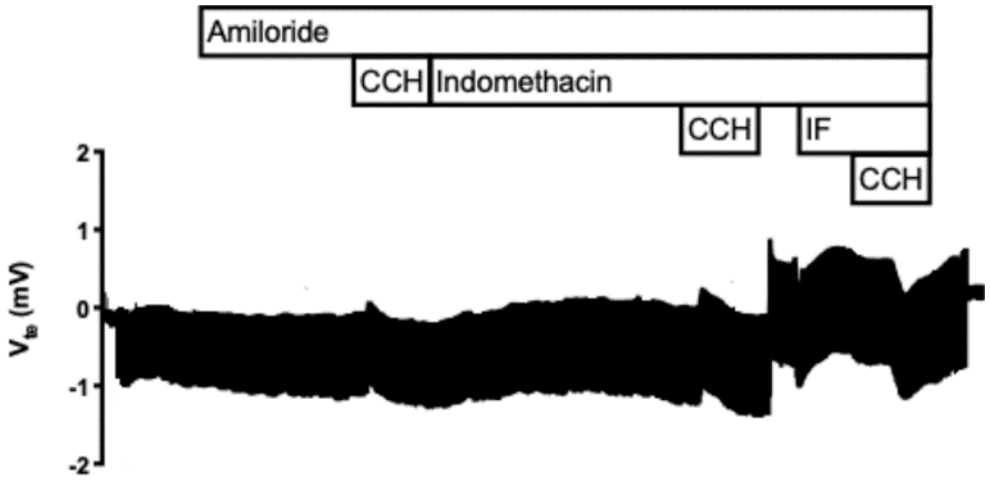
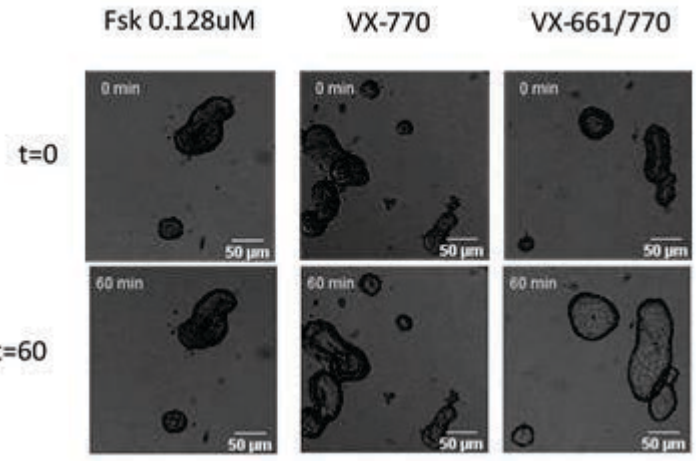


Figure 4.3. Analysis of biopsies from individual 2 (p.Phe508del/p.Arg334Trp) with atypical phenotype shows CFTR residual function.
The graph shows a positive response to IBMX+forskolin (IF), confirming cAMP dependent Cl^- secretion, and Carbachol (CCH) cholinergic co-activation of K^+ channels leading to luminal Cl^- exit. Experiments were performed in the presence of Amiloride (Amil, 20 μM, luminal) and/or Amiloride + Indomethacin (Indo, 10 μM, basolateral), as indicated in the figure. Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.

Drug response

FIS results indicate the presence of residual function in basal conditions, likely associated to the p.Arg334Trp allele. Modest CFTR rescue was observed with the VX-661/VX-770 combination treatment at [Fsk] = 0.128 μM, suggesting potential for clinical benefit. It is thus likely that this individual will benefit from this therapy, although this drug is not currently indicated for individuals with this variant (Figure 4.4).

A



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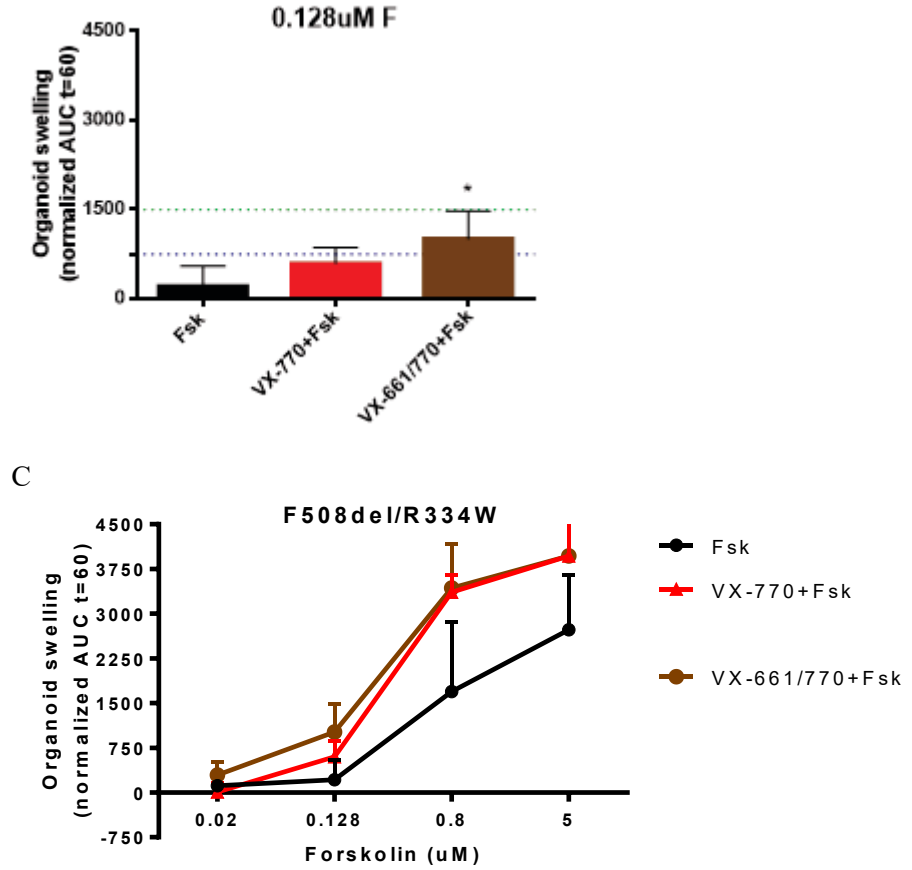


Figure 4.4. Results of the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 2 (*p.Phe508del/p.Arg334Trp *genotype).
(A) Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770 and VX-661/770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min), before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show more swelling with combination therapy VX-770/VX-661. (B) Quantification of organoid swelling for all treatments at [Fsk] = 0.128 µM. The dashed blue and green lines represent the established thresholds for medium and high clinical benefit potential for treatments, respectively. Data represent the mean of measurements on 5-8 replicate wells per condition. (C) Quantification of FIS in organoids for all treatments tested: Fsk alone, VX-770 (3 µM) + Fsk, VX-661/770 at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 µM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.

Individual 3 - p.Arg334Trp / p.Phe508del genotype

Individual 3 is a 9-year-old girl, whose diagnosis of CF was made at the age of 9 months because of an episode of hypochloraemic/hyponatraemic dehydration with metabolic alkalosis, an elevated SCT confirmed the diagnosis. NBS screening was not available when she was born. Her pulmonary function is still normal. She is also PS and adequate weight gain and growth (BMI z-score is 0,9; height z-score is 1).

Intestinal voltage measurements in the Ussing chamber

In individual 3 we found a positive response to IBMX+forskolin (IF), confirming cAMP dependent Cl^- secretion, and Carbachol (CCH) cholinergic co-activation of K^+ channels leading to luminal Cl^- exit. This indicates the presence of partial CFTR function, i.e., residual function (Figure 4.5).

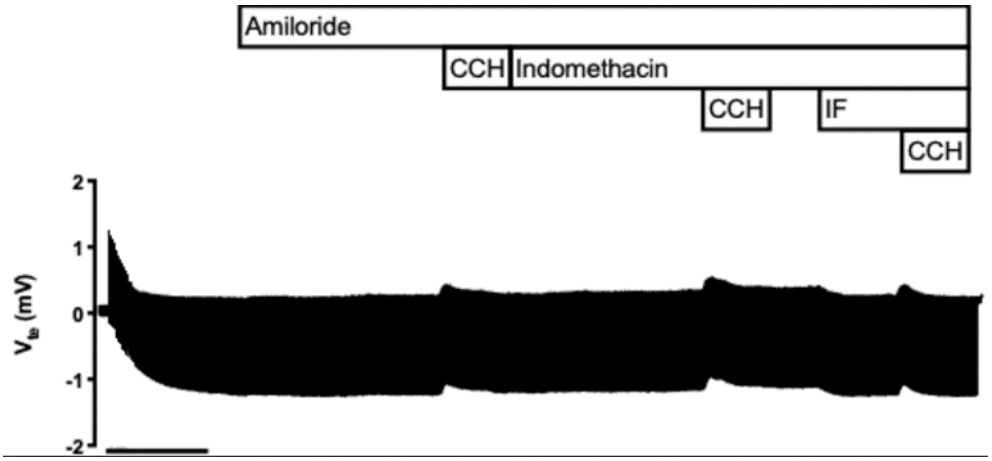


Figure 4.5. Individual 3 (*p.Phe508del/p.Arg334Trp*) with an atypical phenotype has CFTR residual function. *The graph shows a positive response to IBMX+forskolin (IF), confirming cAMP dependent Cl^- secretion, and Carbachol (CCH) cholinergic co-activation of K^+ channels leading to luminal Cl^- exit. Experiments were performed in the presence of Amiloride (Amil, 20 μM, luminal) and/or Amiloride + Indomethacin (Indo, 10 μM, basolateral), as indicated in the figure. Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.*

Drug response

FIS results indicate the presence of residual function in basal conditions, likely associated to the p.Arg334Trp allele. Modest CFTR rescue was observed with the VX-661/VX-770 combination treatment at [Fsk] = 0.128 μM, suggesting potential for clinical benefit. In this individual´s organoids, a triple therapy was also tested and a significant CFTR rescue was found. This suggests that individual 3 should already clinically benefit from combination therapy with VX-661/VX-770770 (Figure 4.6B, brown bar), but a more significant clinical response should be expected from triple therapy with VX-445/VX-661/VX-770 (Figure 4.6B, purple bar).

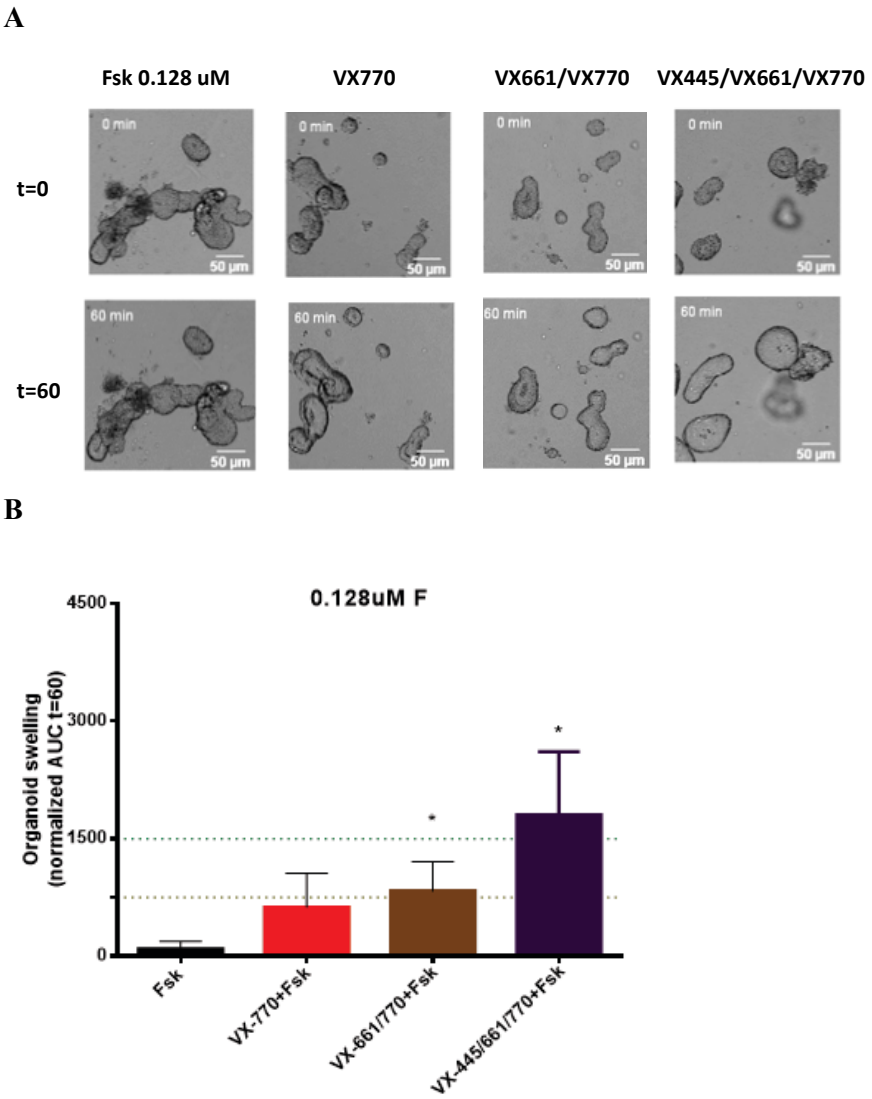


Figure 4.6. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 3 (p.Phe508del/p.Arg334Trp genotype).

(A) *Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770, VX-661/ VX-770 and VX-445/VX-661/VX-770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min) before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show more swelling with combination therapy VX-770/VX-*

661 and triple therapy VX-445/VX-661/VX-770. ***(B)*** *Quantification of organoid swelling for all treatments at [Fsk] = 0.128 μM. The dashed blue and green lines represent the established thresholds for medium and high clinical benefit potential for treatments, respectively. Data represent the mean of measurements on 5-8 replicate wells per condition.* ***(C)*** *Quantification of FIS in organoids for all treatments tested: Fsk alone, VX-770 (3 μM) + Fsk, VX-661/VX-770 + Fsk and VX-445/VX-661/VX-770 + Fsk at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 μM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.*

Individuals with p.Phe508del/ c.579+1G>T genotype

Two pwCF carry the class I variant c.579+1G>T in heterozygosity with p.Phe508del (individuals 4 and 5). c.579+1G>T (frequency of 0.00193[17]) is a CF-causing variant that was shown to change the splice donor site at the 3' end of exon 5, apparently causing its skipping in the alternative transcript and abolishing production of normal transcripts[169]. This variant has been associated to a classic CF phenotype[169].

Individual 4 - p.Phe508del / c.579+1G>T genotype

Individual 4 is a 15-year-old boy, who had meconium ileus at birth which prompted the diagnosis of CF. He still has normal pulmonary function (FEV1 is 107%). He is PI from birth but with a good adherence to PERT, thus contributing to a reasonable nutritional status (BMI z-score is -0.7; height z-score is 1) and normal bone density (Table 4.1). CF related liver disease is present in this individual.

Intestinal voltage measurements in the Ussing chamber

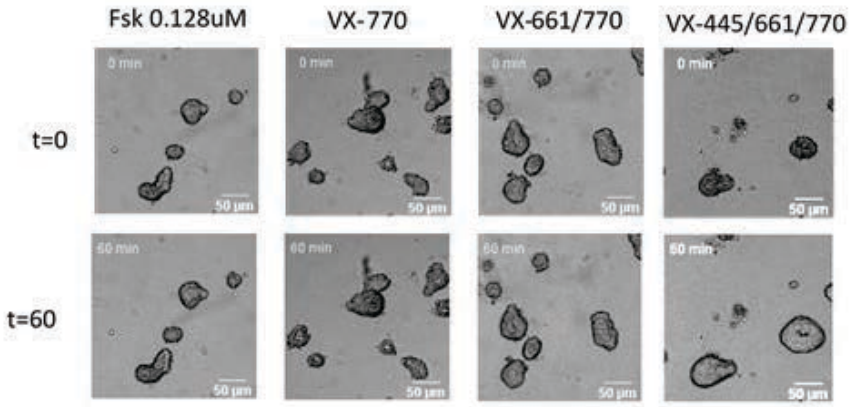
In individual 4, intestinal voltage measurements were not possible to obtain, due to the poor quality of the tissue.

Drug response

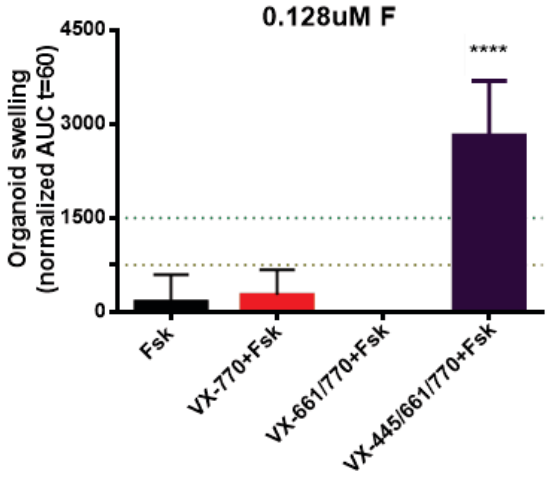
Although Ussing chamber recordings from analyses of rectal biopsies from individual 4 were not obtained, organoid cultures were still produced from his biopsies. FIS results from analyses of organoids from individual 4 indicate no residual function in all control conditions.

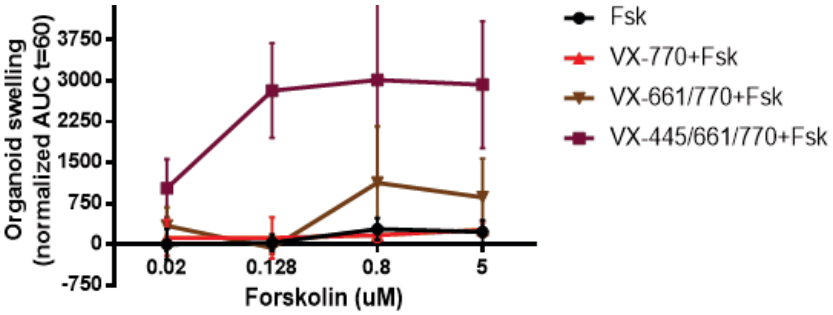
CFTR rescue was not detected with treatment with VX-770 alone nor with VX-661/VX-770 combined therapy, so it is unlikely that this individual will benefit from these therapies. In this individual´s organoids, triple therapy was also tested and significant CFTR rescue was found. This result suggests that this individual should have significant clinical benefit from the triple therapy with VX-445/VX-661/VX-770 (purple bar in Figure 4.7B), as expected due to the presence of the p.Phe508del variant.

A


Fsk 0.128uM
VX-770
VX-661/770
VX-445/661/770
t=0
t=60
0 min
60 min
50 µm

B


0.128uM F
Organoid swelling (normalized AUC t=60)
4500
3000
1500
0
****
Fsk
VX-770+Fsk
VX-661/770+Fsk
VX-445/661/770+Fsk



C

Figure 4.7. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 4 (p.Phe508del/711+1G>T genotype).

(A) *Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770, VX-661/VX-770 and VX-445/VX-661/VX-770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min) before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show more swelling with combination therapy VX-770/VX-661 and triple therapy VX-445/VX-661/ VX-770.* ***(B)*** *Quantification of organoid swelling for all treatments at [Fsk] = 0.128 µM. The dashed blue and green lines represent the established thresholds for medium and high clinical benefit potential for treatments, respectively. Data represent the mean of measurements on 5-8 replicate wells per condition.* ***(C)*** *Quantification of FIS in organoids for all treatments tested: Fsk alone, VX-770 (3 µM) + Fsk, VX-661/ VX-770 + Fsk and VX-445/ VX-661/ VX-770 + Fsk at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 µM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.*

Individual 5 - p.Phe508del / c.579+1G>T genotype

Individual 5 is a 12-year-old girl, whose diagnosis was obtained at 1 month old. She has a severely compromised lung function as demonstrated by her FEV1 of 30%. She is PI; has a poor nutritional status (BMI z-score is -2 and height z-score is -1.8); CF-related bone disease and CF-related liver disease (Table 4.1).

Intestinal voltage measurements in the Ussing chamber

In rectal biopsies from individual 5, no response was observed to CFTR inducers IBMX + Fsk or CCH was obtained, which means that no Cl^- was secreted. On the contrary, a positive peak is found due to the isolated secretion of K^+. This allowed us to conclude that there is no CFTR function (some authors call this 'minimal' function but we do not use this terminology because it is confounding with 'residual function') (Figure 4.8).

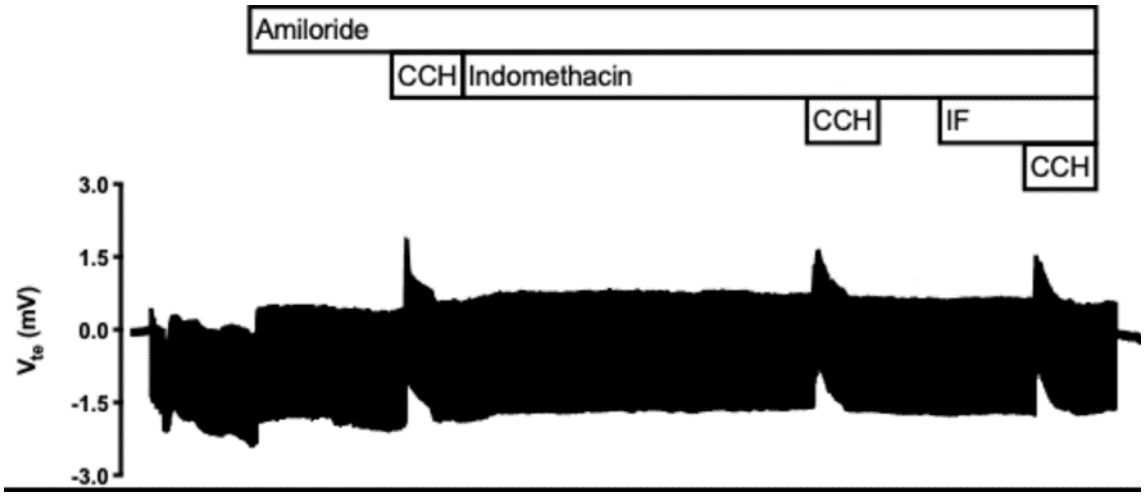


Figure 4.8. Results from Ussing chamber measurements in rectal biopsies from individual 5 (p.Phe508del***/c.579+1G>T genotype).***
The graph shows no response to IBMX+forskolin (IF), confirming no cAMP dependent Cl^- secretion, and no Carbachol (CCH) cholinergic co-activation of K^+ channels on transepithelial voltage (Vte). Experiments were performed in the presence of Amiloride (Amil, 20 μM, luminal) and/or Amiloride + Indomethacin (Indo, 10 μM, basolateral), as indicated in the figure. Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.

Drug response

FIS results in organoids from individual 5, CFTR rescue was not detected with treatment with VX-770 alone nor with VX-661/VX-770 combined therapy, so it is unlikely that this individual will benefit from these therapies. In this individual´s organoids, triple therapy was also tested and a significant CFTR rescue was found. This suggests that this individual should have significant clinical benefit from the triple therapy with VX-445/VX-661/VX-770 (Figure 4.9B, burgundy bar).

This individual started on triple therapy at the age of 13 years, and 6 months later, sweat Cl^- concentration was practically on the normal range (45 μmol/l), BMI z-score increased from -1,81 to -0,46 due to weight gain, however her height z-score that did not improve. Her pulmonary function was already severely compromised when she started therapy but it still showed a modest improvement: FEV1 increased from 30% to 34%.

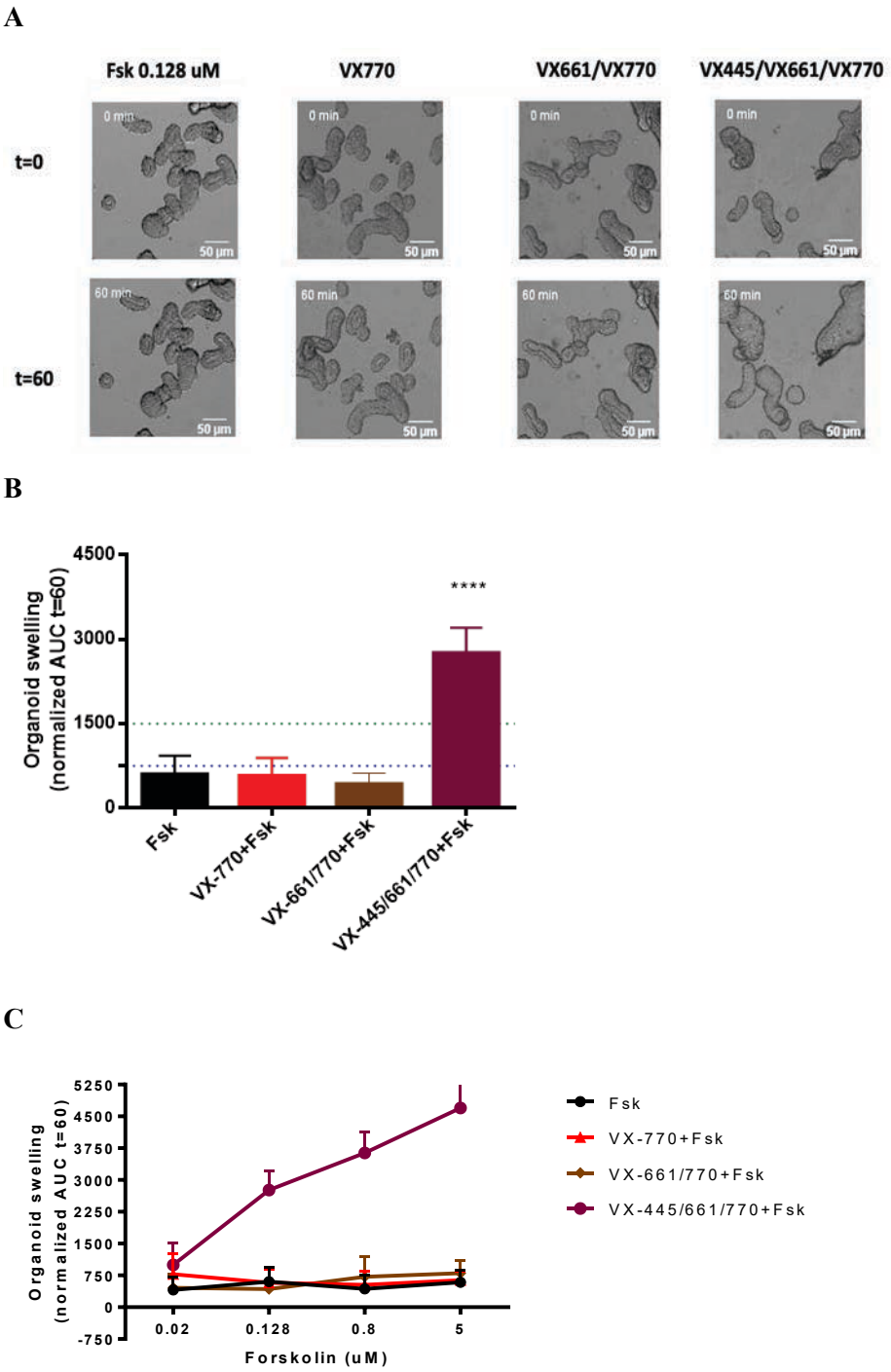


Figure 4.9. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 5 (*p.Phe508del*/711+1G>T genotype

(A) *Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770, VX-661/ VX-770 and VX-445/VX-661/VX-770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min), before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show more swelling with combination therapy VX-770/VX-661 and triple therapy VX-445/ VX-661/VX-770.* ***(B)*** *Quantification of organoid swelling for all treatments at [Fsk] = 0.128 µM. The dashed blue and green lines represent the established thresholds for medium and high clinical benefit potential for treatments, respectively. Data represent the mean of measurements on 5-8 replicate wells per condition.* ***(C)*** *Quantification of FIS in organoids for all treatments tested: Fsk alone, VX-770 (3 µM) + Fsk, VX-661/VX-770 + Fsk and VX-445/ VX-661/ VX-770 + Fsk at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 µM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.*

Individual 6 - p.Phe508del/ p.Gln685ThrfsX4 genotype

Individual 6 (female) had the diagnosis of CF at 2 years old in the context of recurrent pneumonias. She has a severe phenotype characterized by PI, severe malnutrition (BMI z-score is -4,9; height z-score is -2.71), severely compromised pulmonary function (FEV1 = 25%) and CF-related bone disease (Table 4.1).

She carries a class VII variant, p.Gln685ThrfsX4. This rare variant with a frequency of 0.00232[17], is a CF-causing variant[17]. This variant has been associated to a classic CF phenotype[170].

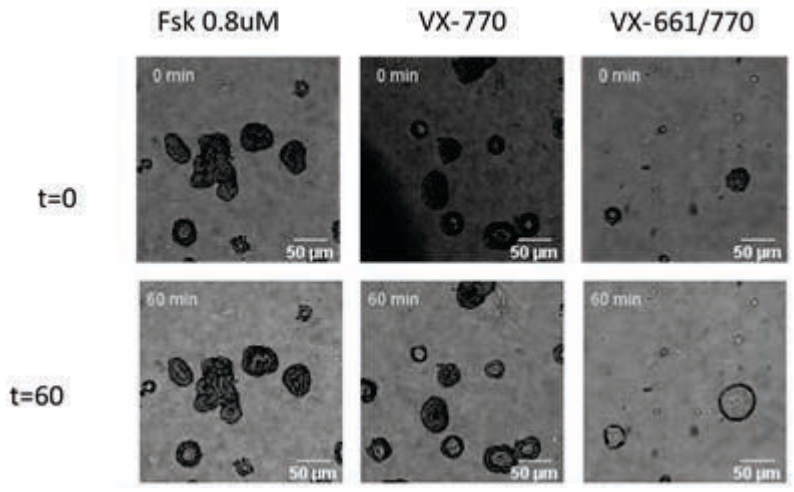
Intestinal voltage measurements in the Ussing chamber

Intestinal voltage measurements were not possible to obtain in biopsies from individual 6, due to the low quality of the tissue.

Drug response

FIS results from analysis of organoids from individual 6 indicate absence of CFTR function in all control conditions. CFTR rescue was not detected with any of the tested treatments (VX-770 and VX-661/VX-770), so it is unlikely that this individual will benefit from any of these therapies (Figure 4.10B, red and brown lines, respectively). Triple therapy was not tested in this individual, although she would likely respond positivey, given the presence of the p.Phe508del variant.

A



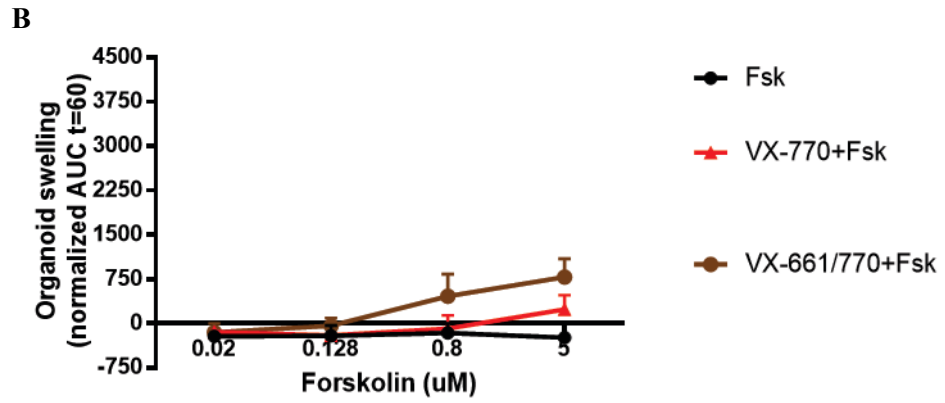


Figure 4.10. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 6 (p.Phe508del/p.Gln685ThrfsX4 genotype).
(A) Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128μM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770 and VX-661/ VX-770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min), before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show no swelling with any drug. ***(B)*** *Quantification of FIS in organoids for all treatments tested: Fsk alone, VX-770 (3 μM) + Fsk, VX-661/ VX-770 at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 μM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.*

Individual 7 - p.Phe508del/ c.3321dup genotype

Individual 7 is a 2-year-old boy who carries a new variant c.3321dup that was not previously described. This variant is most likely pathogenic because it causes a frameshift after Trp1063 and therefore it can be considered Class VII. This individual was born with meconium ileus and the NBS was unexpectedly negative. However the values from the SCT, performed with one month of age, confirmed the diagnosis of CF. He is PI. Because of poor weight gain he has enteral nutrition support by gastrostomy. He also has CF-related diabetes treated with insulin and has severe pulmonary manifestations requiring nocturnal non-invasive ventilation with supplemental oxygen and prolonged admissions to the hospital since birth.

Intestinal voltage measurements in the Ussing chamber

Intestinal voltage measurements were not possible to obtain in biopsies from individual 7, due to the poor quality of the tissue.

Drug response

FIS results from analyses of organoids from individual 7 evidence absence of CFTR function in all basal conditions. CFTR rescue was not detected with any treatment tested.

However, given the presence of the p.Phe508del variant, he would likely respond to the triple combination, but this was not tested. Thus, it is highly unlikely that this individual would benefit from the two above treatments (Figure 4.11B), red and brown lines, respectively).

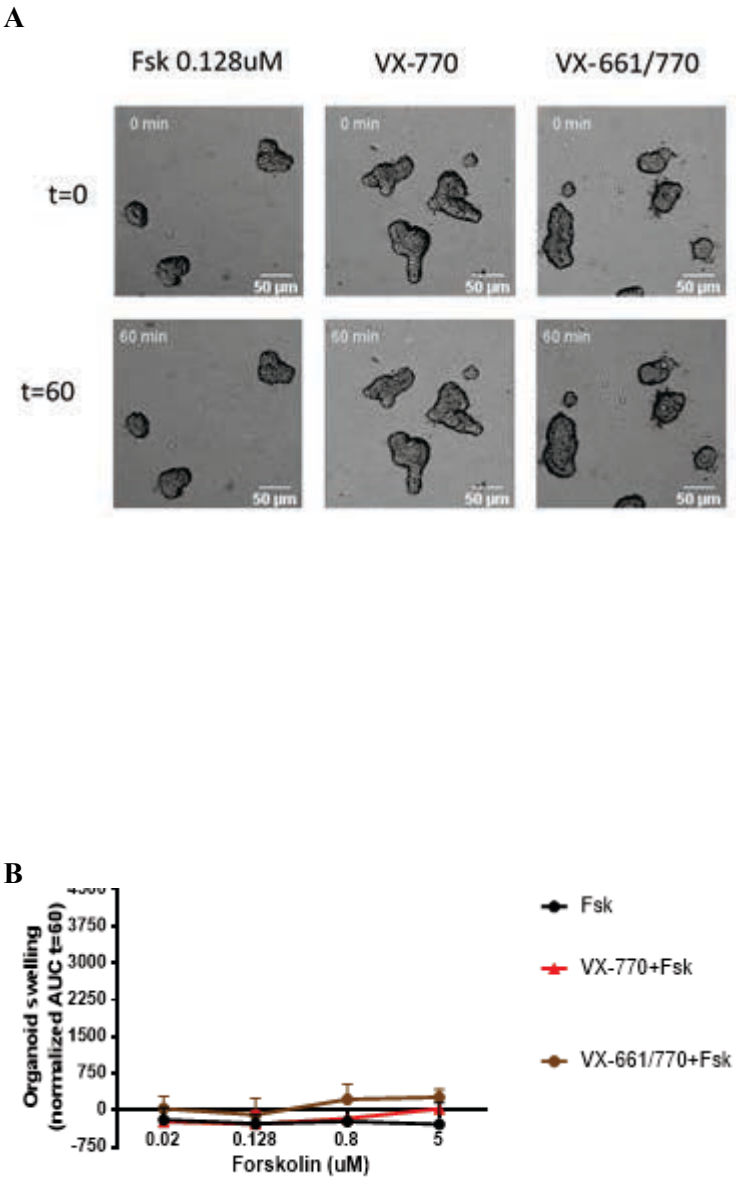


Figure 4.11. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 7 (*p.Phe508del*/ c.3321dup genotype).

(A) *Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770 and VX-661/ VX-770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min) before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show no swelling with any drug.* ***(B)*** *Quantification of FIS in organoids for all treatments tested: Fsk alone, VX-770 (3 µM) + Fsk, VX-661/ VX-770 at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 µM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.*

Individual 8 - p.Phe508del / p.Pro5Leu genotype

Individual 8 is a two-year-old boy who carries the unclassified p.Pro5Leu variant. He was diagnosed through a positive NBS test. At 2 months of age, two independent SCT were positive (89 mmol/L and 85 mmol/L). Genetic testing confirmed the presence of the most common disease causing variant - p.Phe508del - and of another rare variant - p.Pro5Leu. The p.Pro5Leu variant (legacy name P5L) consists of an amino acid change (proline by a leucine) at the N-terminus, i.e., at the CFTR cytoplasmic tail, caused by a C>T substitution at the cDNA nucleotide position c.14, located in exon 1, at codon five [171].

Unlike other N-terminus missense variants which cause a CFTR folding/ trafficking defect affecting CFTR conformation and causing classical forms of CF, p.Pro5Leu seems to be associated to an atypical CF phenotype [171]. p.Pro5Leu variant has been described as exhibiting features of both a folding mutant and of a defective channel. Experimental immunodetection data have showed a small but significant amount of mature p.Phe5Leu CFTR mutant at the PM, not corroborating a previous report classifying p.Phe5Leu variant as a class II variant [171,172]. Electrophysiological analyses of the mature p.Pro5Leu also revealed altered channel gating [172].

p.Pro5Leu is described in the CFTR2 variant database has a variant with varying consequences, meaning that when combined with another CF-causing variant, may or may not cause CF. Because of this variability it is recommended that clinical criteria alone is used to determine the diagnosis. [75]

This individual is PS and is clinically asymptomatic. No respiratory symptoms, no chronic pulmonary infections and adequate nutritional status (Table 4.1**Erro! A origem da referência não foi encontrada.**).

Intestinal voltage measurements in the Ussing chamber

In biopsies from individual 8 we found a positive response to IBMX+Fsk, confirming cAMP- dependent Cl^- secretion, and Carbachol (CCH) cholinergic co-activation of K^+ channels leading to luminal Cl^- exit. This indicates the presence of partial CFTR function, i.e., residual function (Figure 4.12).

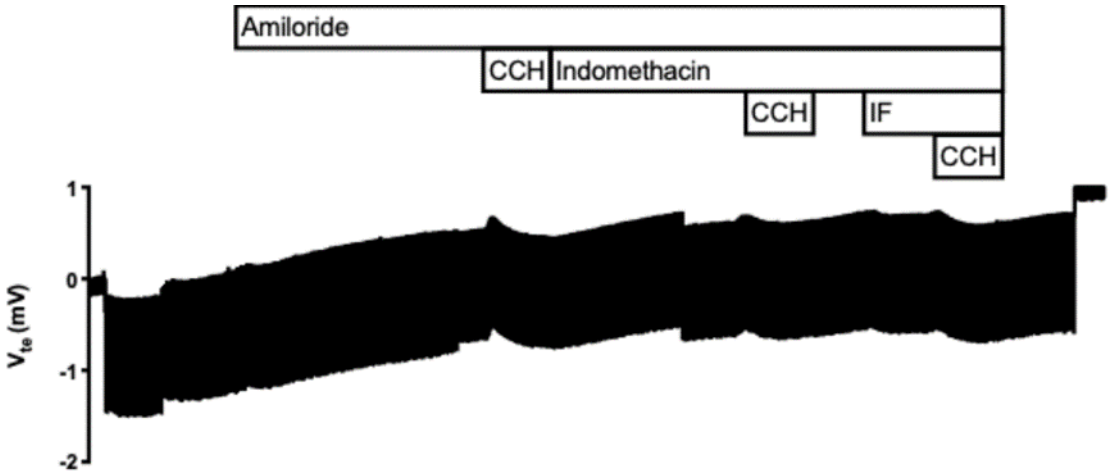
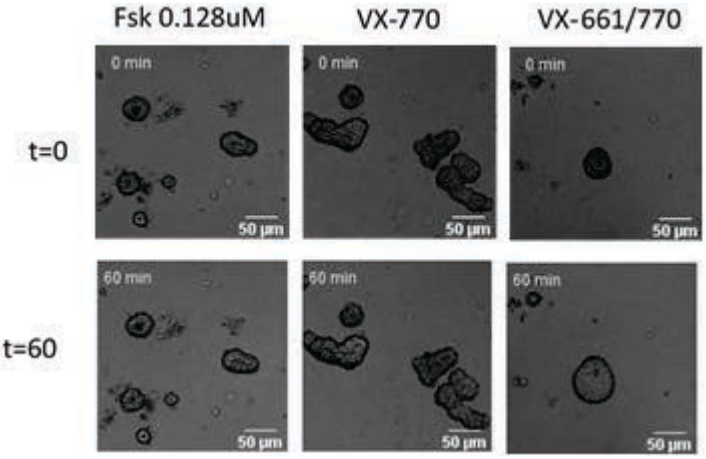


Figure.4.12. Individual 8 (p.Phe508del/p.Pro5Leu) with atypical phenotype has CFTR residual function.
The graph shows a positive response to IBMX+forskolin (IF), confirming cAMP dependent Cl^- secretion, and Carbachol (CCH) cholinergic co-activation of K^+ channels leading to luminal Cl^- exit. Experiments were performed in the presence of Amiloride (Amil, 20 µM, luminal) and/or Amiloride + Indomethacin (Indo, 10 µM, basolateral), as indicated in the figure. Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.

Drug response

Results from FIS analyses of intestinal organoids from this individual indicate the presence of residual function in basal conditions. Significant CFTR rescue was detected with VX-661/VX-770 combined treatment at [Fsk] = 0.128 µM suggesting a potential clinical benefit (Figure 4.13B, brown bar), so it is likely that this individual will benefit from this therapy.

A



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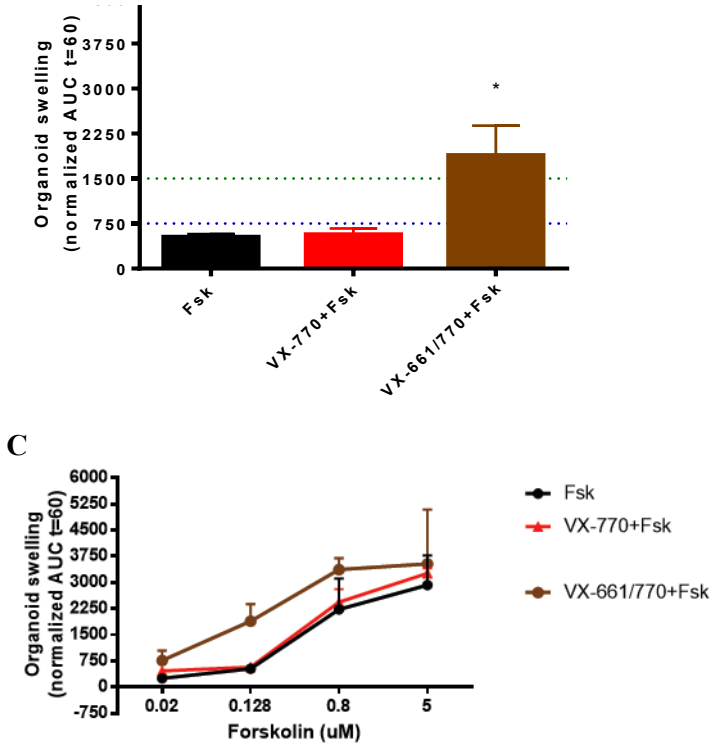


Figure 4.13. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 8 (p.Phe508del***/p.Pro5Leu genotype).***
(A) *Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770 and VX-661/ VX-770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min) before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show more swelling with combination therapy VX-770/VX-661.* ***(B)*** *Quantification of organoid swelling for all treatments at [Fsk] = 0.128 µM. The dashed blue and green lines represent the established thresholds for medium and high clinical benefit potential for treatments, respectively. Data represent the mean of measurements on 5-8 replicate wells per condition.* ***(C)*** *Quantification of FIS in organoids for all treatments tested: Fsk alone, VX-770 (3 µM) + Fsk, VX-661/ VX-770 at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 µM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.*

People with CF with p.Phe508del / p.Phe508del genotype

Three individuals analysed in this study were p.Phe508del-CFTR homozygous, namely individuals 9, 10 and 11. As previously mentioned, p.Phe508del, the most common variant in pwCF (allele frequency is 0.697[17]), has been extensively characterized as a class II processing mutant with class III/VI defects when correctly transported to the PM. This variant is typically associated to classical forms of CF, characterized by elevated levels of sweat Cl^- and severe pulmonary disease and PI[173].

Table 4.2. Clinical characteristics of 3 pwCF with the p.Phe508del/p.Phe508del genotype

	p.Phe508del/ p.Phe508del
	Class II

Individual number	9	10	11
Gender	M	M	M
Age (years)	9	6	7
Meconium ileus	no	no	no
Neonatal screening[a]	-	-	-
Age at diagnosis	21m	12m	5m
Sweat test[b] **(mmol/L)**	125	108	106
Pancreatic function	PI	PI	PI
BMI z-score	-2.3	-2.6	-0.3
Height z-score	-1	1	0.3
CFRLD	no	no	no
CFRD	no	no	no
Bone density[c]	-0.6	***	***
FEV1 %[d]	95	85	78

[a]Available after 2013
[b]Sweat conductivity test
[c]Lumbar DEXA scan. Usually, performed in children older than 8-9 years old.
[d]Pulmonary function test is only performed in children older than 6 years old

BMI, body mass index; CFRD, CF related diabetes; CFRLD, CF related liver disease; F, female; FEV1, forced expiratory volume in 1 second; M, male; m, months; NA, not applicable; PI, pancreatic insufficient; PS, pancreatic sufficient; y-years.

Individual 9 - p.Phe508del/p.Phe508del

Individual 9 (male) had persistent wheezing and poor weight gain in the first years of life and he was diagnosed with CF at 21 months. He is PI and has poor nutritional status (BMI z-score is -2.3). Pulmonary function, however, is within normal range (Table 4.2).

Intestinal voltage measurements in the Ussing chamber

Intestinal voltage measurements were not possible to obtain in the rectal biopsies of individual 9, due to the poor quality of the tissue.

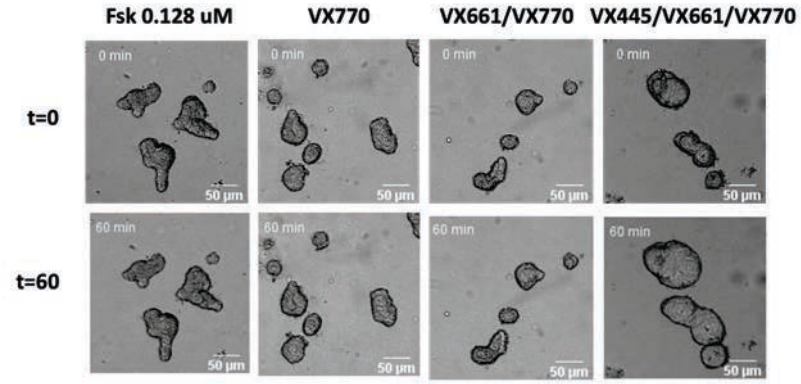
Drug response

Results from FIS analyses in intestinal organoids from this individual indicate the complete absence of CFTR function in basal conditions. No statistically significant CFTR rescue was detected with combination treatment with VX-661/VX-770 at [Fsk] = 0.128

µM (Figure 4.14B, brown bar). Even though this therapy is indicated for individuals with this genotype, this particular individual does not seem to clinically benefit from it.

In contrast, significant CFTR rescue was obtained with triple therapy (Figure 4.14B, burgundy bar). It is thus likely that this individual will have significant clinical benefit from the triple therapy.

A



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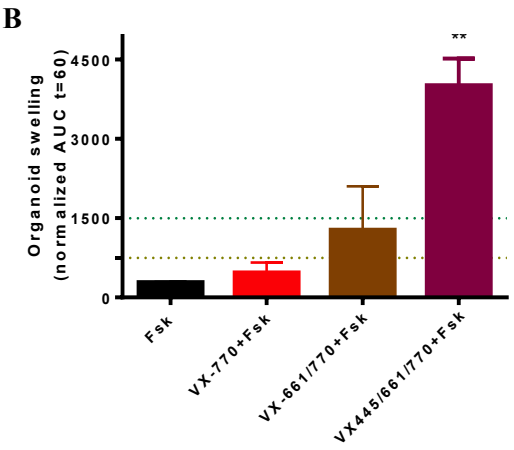

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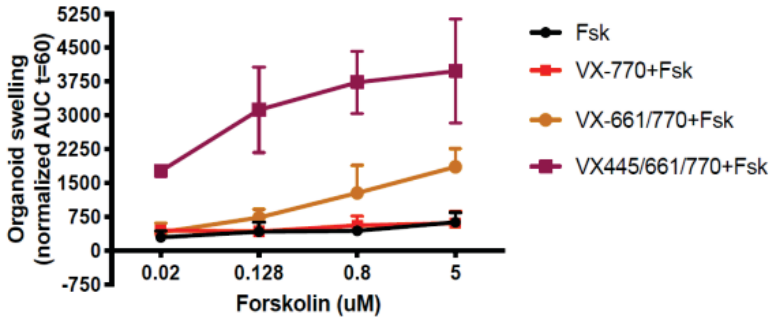

Figure 4.14. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 9 (p.Phe508del/p.Phe508del ***genotype).***
(A) Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770, VX-661/VX-770 and VX-445/VX-661 /VX-770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min, before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show more swelling with triple therapy VX-445/VX-661/VX-770. (B) Quantification of organoid swelling for all treatments at [Fsk] = 0.128 µM. The dashed blue and green lines represent the established thresholds for medium and high clinical benefit potential for treatments, respectively. Data represent the mean of measurements on 5-8 replicate wells per condition. (C) Quantification of FIS in organoids for all treatments tested (Forskolin (Fsk) alone, VX-770 (3 µM) + Fsk, VX-661/ VX-770, VX-445/VX-661/ VX-770 at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 µM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.

Individual 10 - p.Phe508del/p.Phe508del

This male individual had persistent wheezing and poor weight gain in the first year of life and he was diagnosed with CF at 12 months. He is PI and he has a poor nutritional status (BMI z-score is -2.6). Pulmonary function is within normal range (Table 4.2).

Intestinal voltage measurements in the Ussing chamber

Intestinal voltage measurements were not possible to obtain in rectal biopsies from individual 10, due to the poor quality of the tissue.

Drug response

Intestinal organoids of this individual were not viable either.

Individual 11 - p.Phe508del/p.Phe508del

Individual 11 had persistent wheezing and chronic cough, weight gain was poor, and he was diagnosed with CF at 5 months. He is PI and he now has an adequate nutritional status (BMI z-score is -2.3 and height z-score is 0.3). Pulmonary function is within normal range (Table 4.2).

Intestinal voltage measurements in the Ussing chamber

The profile of the representative Ussing chamber tracings from the analysis of rectal biopsies from individual 11 indicate that these samples are from a pwCF carrying a *CFTR* genotype and that may present classical CF form of CF disease. Indeed, the tracings clearly evidence a positive K^+ secretory response to CCH and cholinergic co-activation of the tissues, while these also show no basal CFTR function (Figure 4.15).

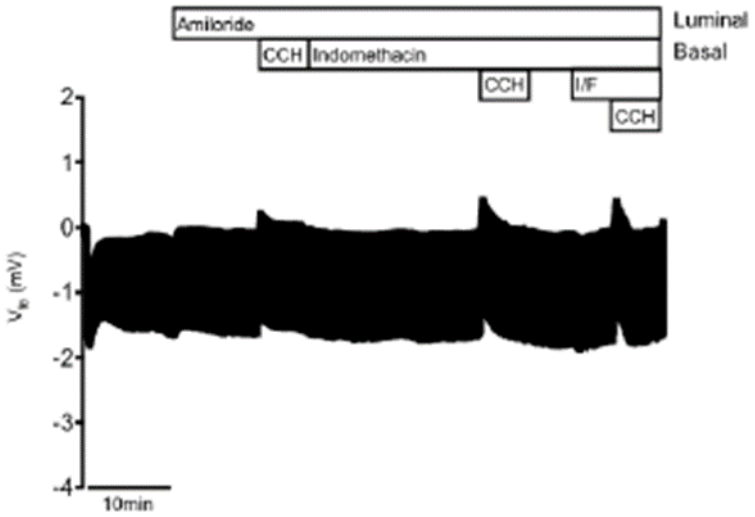

Figure 4.15. Results from Ussing chamber measurements in rectal biopsies from individual 11 (p.Phe508del/p.Phe508del ***genotype) shows no CFTR function.***
The graph shows no response to IBMX+forskolin (IF), confirming no cAMP dependent Cl^- secretion, and no Carbachol (CCH) cholinergic co-activation of K^+ channels on transepithelial voltage (Vte). Experiments were performed in the presence of Amiloride (Amil, 20 μM, luminal) and/or Amiloride + Indomethacin (Indo, 10 μM, basolateral), as indicated in the figure. Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission .

Drug response

In similarity with individual 9, results from FIS analyses in intestinal organoids from this individual indicate the complete absence of CFTR function in basal conditions. CFTR rescue was not significant with the VX-661/VX-770 combined treatment at [Fsk] = 0.128 μM suggesting that clinical benefit is unlikely (Figure 4.16B, red bar). Even though this therapy is indicated for individuals with this genotype, this particular individual does not seem to obtain significant clinical benefit from it.

However, significant CFTR rescue was obtained with the triple therapy suggesting a high clinical benefit from this therapy (Figure 4.16B, burgundy bar).

However, we can detect a slight difference between responses in organoids from the two individuals (9 and 11) sharing the same genotype. Even though, CFTR in both organoids is rescued by the triple therapy, in organoids from individual 9, the response is slightly

higher. The same can be found for the double combination therapy. Theoretically, we can expect a better clinical response with both therapeutic options for individual 9, in comparison to individual 11, despite the same genotype. However, given the small differences observed between the two responses, this may not be the case.

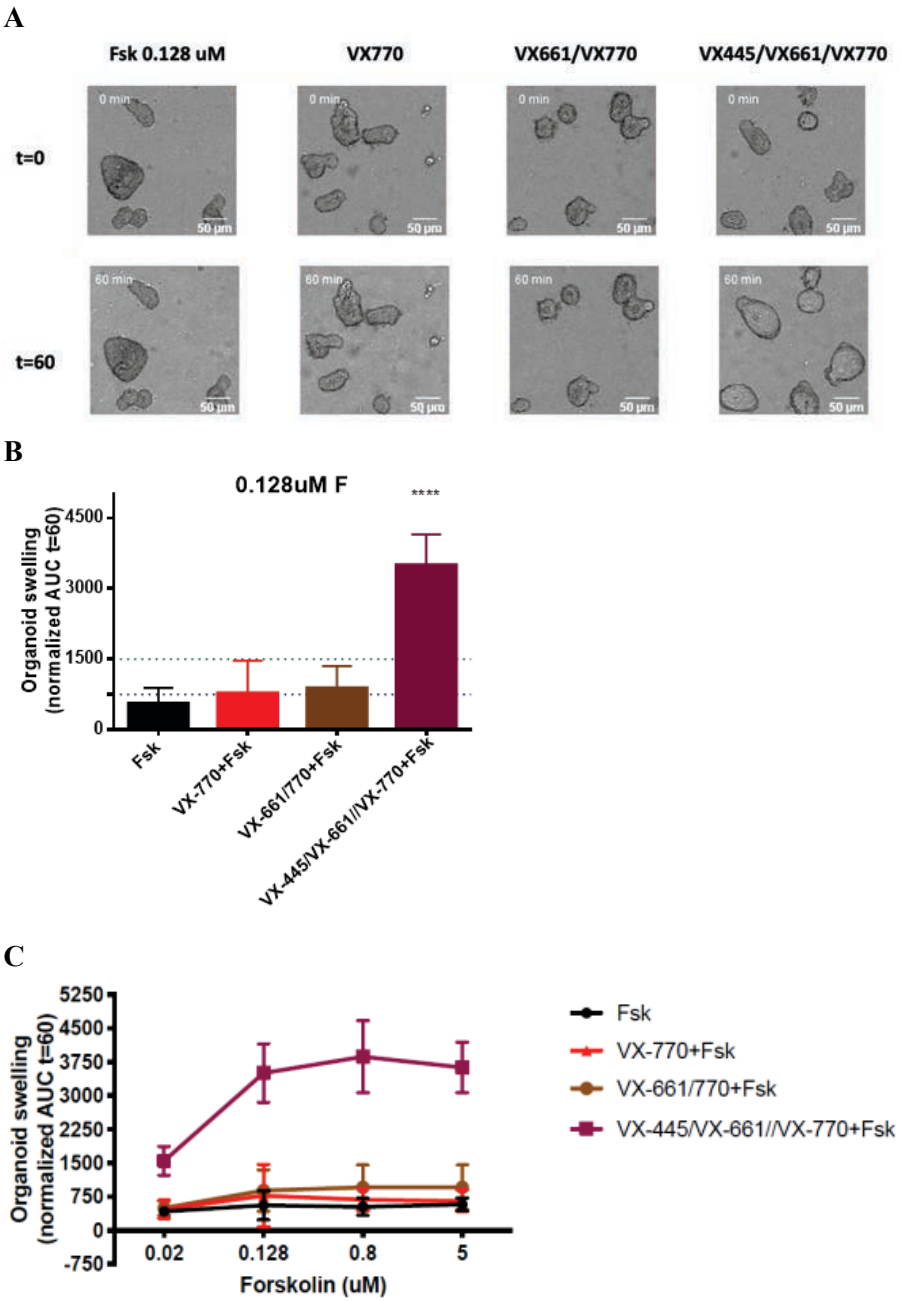

Figure 4.16. Results from the forskolin-induced swelling (FIS) assay on intestinal organoids from individual 11 (p.Phe508del/p.Phe508del ***genotype).***

(A) *Organoid images before and after stimulation with forskolin (Fsk) at the concentration of 0.128µM first without any treatment (left panels) and after incubation with CFTR modulators: VX-770, VX-661/ VX-and VX-445/ VX-661/ VX-770, as indicated above each panel. Images from superior panels show organoids at the beginning of the experiment (t=0 min) before stimulation with Fsk. Inferior panels show organoids at the end of the experiment (t=60 min) which means 60 min after stimulation with Fsk. Results show more swelling with combination therapy VX-770/VX-661 and triple therapy VX-445/ VX-661/ VX-770.* ***(B)*** *Quantification of organoid swelling for all treatments at [Fsk] = 0.128*

μM. The dashed blue and green lines represent the established thresholds for medium and high clinical benefit potential for treatments, respectively. Data represent the mean of measurements on 5-8 replicate wells per condition. ***(C)*** *Quantification of FIS in organoids for all treatments tested (Forskolin (Fsk) alone, VX-770 (3 μM) + Fsk, VX-661/VX-770, and VX-445/ VX-661/ VX-770 at the Fsk concentrations of 0.02, 0.128, 0.8 and 5 μM, expressed as the AUC of organoid surface area increase (baseline = 100%, t = 60 min). Data generated by I. Silva and J. Roda at the Amaral lab and included here with permission.*

Table 4.3 presents a summary of the results obtained from analyses of intestinal organoids for all pwCF studied here.

Table 4.3. Summary of results from intestinal organoids for all subjects studied (N=11[1]): CFTR genotypes and qualitative CFTR rescue by different modulator drugs.

Individuals	CFTR gene variant	Residual CFTR protein function	Iva	Iva/Teza	Elexa/Teza/ Iva
1	p.Arg334Trp	yes	*	*	nt
2	p.Arg334Trp	yes	ns	*	nt
3	p.Arg334Trp	yes	ns	*	*
4	c.579+1G>T	no	ns	ns	***
5	c.579+1G>T	no	ns	ns	***
6	p.Gln685ThrfsX4	no	ns	ns	nt
7	c.3321dup	no	ns	ns	nt
8	p.Pro5Leu	yes	ns	*	nt
9	p.Phe508del/p.Phe 508del	no	ns	ns	**
10	p.Phe508del/p.Phe 508del	-	-	-	-
11	p.Phe508del/p.Phe 508del	no	ns	ns	***

Asterisks (*) indicate the degree of statistical significance of detected difference to control.
ns, not significant; nt, not tested.
[1]Although 11 pwCF were selected for this study, only results from 10 were obtained

Correlations among different CFTR function parameters, established CF biomarkers and clinical data

Correlation analyses were performed among different CFTR function parameters used in this project, namely the intestinal voltage measurements (IVM) used as a readout for CFTR function in rectal biopsies. Voltage measurements were converted into equivalent currents by the Ohm's law, namely: the cAMP-mediated activated current = $\Delta I_{eq\text{-}sc}(I/F)$, the cholinergic co-activated current = $\Delta I_{eq\text{-}sc}(CCH(I/F))$ and the sum of these two currents,

corresponding to the maximal CFTR activation = $\Delta I_{eq\text{-}sc}$(max)). These were correlated with the individual-matched intestinal organoid FIS readouts at the different Fsk concentrations (0.02, 0.128, 0.8 and 5 μM), in basal conditions (no treatment). The intestinal organoid FIS assay is a fairly recent approach for CFTR function analysis that is currently used for the prediction of each individualized treatment benefit, based on the response to CFTR-modulator drugs in his/her own organoids. However, these measurements are not validated as a diagnostic and prognostic tool for CF, contrarily to the IVM.

These analyses showed that no significant correlations were found between IVM and FIS assay in intestinal organoids of the same individual (Table 4.4).

Table 4.4. Correlation between cAMP- and cholinergic-mediated equivalent short circuit currents in rectal biopsies and FIS in organoids.

Correlation analyses were established between parameters 1 and 2. Parameter 1 referers to equivalent short circuit currents in rectal biopsies upon addition of IBMX/Fsk ($\Delta I_{eq\text{-}sc}$(I/F)), CCH in the presence of IBMX/Fsk ($\Delta I_{eq\text{-}sc}$(CCH(I/F))) and the sum of these two currents, corresponding to the maximal CFTR activation ($\Delta I_{eq\text{-}sc}$(max)) (μA/cm²). Parameter 2 relates to organoid swelling at the different Fsk concentrations tested in the FIS assay (0.02, 0.128, 0.8 and 5 μM) and in control conditions (no treatment). Pearson correlation coefficients (r), respective p-values and sample sizes (n) are indicated. Significant p-values (<0.05) are highlighted in bold.

Parameter 1	Parameter 2	Pearson r	p-value	n
ΔIeq-sc(I/F)	FIS 0.02	0.576	0.310	5
	FIS 0.128	0.414	0.488	5
	FIS 0.8	-0.416	0.486	5
	FIS 5	-0.4	0.505	5
ΔIeq-sc (CCH(I/F))	FIS 0.02	0.130	0.835	5
	FIS 0.128	0.001	0.999	5
	FIS 0.8	-0.402	0.500	5
	FIS 5	-0.370	0.54	5
ΔIeq-sc(max)	FIS 0.02	0.213	0.73	5
	FIS 0.128	0.076	0.903	5
	FIS 0.8	-0.413	0.49	5
	FIS 5	-0.383	0.52	5

Correlation analyses were also performed between CFTR function as determined from ICM and FIS readouts and other established CF-characteristic biomarkers and clinical data that are indicative of the disease severity, namely the SCT values, BMI and of FEV1 values (Table 4.5). These analyses also showed no significant correlations between the values from the SCT, FEV1 and BMI biomarkers with IVM (Table 4.5). Nonetheless, it should be mentioned these analyses were performed in a small number of individuals (n=5) for SCT and BMI and (n=4) for FEV1 (which cannot be performed in children younger than six years.

Furthermore, the same analyses showed that the SCT values significantly correlated with individual-matched organoid swelling for [Fsk] = 0.8 and 5 μM concentrations, and this was higher for [Fsk] = 0.8 μM (r = -0.753, p = 0.012, n = 10) (Table 4.5). On the other hand, FEV1 and BMI values did not significantly correlate to the FIS values in organoids for any of the Fsk concentrations used, probably due to the fact that age stratification was not possible with the low number of individuals analysed here.

Overall, these results indicate that the *in vitro* organoid swelling in basal conditions of CFTR activation (no treatment) at the Fsk concentration of 0.8 μM evidences a stronger correlation with the sweat [Cl^-]. Samples from individuals with higher levels of organoid swelling (i.e., more CFTR function) generally corresponded to lower values of SCT which may support an atypical phenotype for these individuals.

Table 4.5. Correlation between cAMP- and cholinergic-mediated equivalent short circuit currents in rectal biopsies and FIS in organoids with CF outcome parameters.

Correlation analyses were established between parameters 1 and 2 and parameters 1 and 3. Parameter 1 refers to CF outcome parameters: sweat [Cl^-] (mmol/L), faecal elastase E1 values (FEE, µg/g stool) and forced expiratory volumes in 1 sec in percentage predicted (FEV_1%). Parameter 2 relates to activated equivalent short circuit currents in rectal biopsies upon addition of IBMX/Fsk ($\Delta I_{eq\text{-}sc}$(I/F)), CCH in the presence of IBMX/Fsk ($\Delta I_{eq\text{-}sc}$(CCH(I/F))) and the sum of these two currents, corresponding to the maximal CFTR activation ($\Delta I_{eq\text{-}sc}$(max)) (µA/cm2). Parameter 3 relates to organoid swelling at the different Fsk concentrations tested in the FIS assay (0.02, 0.128, 0.8 and 5 µM) in control conditions (no treatments). Pearson correlation coefficients (r), respective p-values and sample sizes (n) are denoted. Significant p-values (<0.05) are highlighted in bold and with.*

Clinical data	**Equivalent short circuit currents**				**Organoid swelling**			
Parameter 1	**Parameter 2**	**Pearson r**	**p-value**	**n**	**Parameter 3**	**Pearson r**	**p-value**	***n***
Sweat [Cl^-]	ΔIeq-sc(I/F)	0,653	0.232	5	FIS 0.02	0.295	0.408	10
	ΔIeq-sc(CCH(I/F))	0.600	0.285	5	FIS 0.128	-0.398	0.255	10
	ΔIeq-sc(max)	0.621	0.264	5	FIS 0.8	**-0.753**	**0.012***	10
					FIS 5	**-0.664**	**0.036***	10
FEV1	ΔIeq-sc(I/F)	-0.634	0.366	4	FIS 0.02	0.296	0.476	10
	ΔIeq-sc(CCH(I/F))	-0.812	0.188	4	FIS 0.128	0.267	0.523	10
	ΔIeq-sc(max)	-0.797	0.2	4	FIS 0.8	0.439	0.277	10
					FIS 5	0.509	0.197	10
BMI z-score	ΔIeq-sc(I/F)	-0.731	0.16	5	FIS 0.02	0.294	0.41	10
	ΔIeq-sc(CCH(I/F))	-0.863	0.06	5	FIS 0.128	0.258	0.472	10
	ΔIeq-sc(max)	-0.856	0.064	5	FIS 0.8	0.421	0.225	10
					FIS 5	0.421	0.432	10

A significant difference was found between all IVM used for a readout of CFTR function in individuals who are PS and PI (Table 4.6).

Table 4.6. Comparison of cAMP- and cholinergic-mediated equivalent short circuit currents in rectal biopsies between pwCF who are PS and PI.
Parameter 2 relates to activated equivalent short circuit currents in rectal biopsies upon addition of IBMX/Fsk ($\Delta I_{eq\text{-}sc}$(I/F)), CCH in the presence of IBMX/Fsk ($\Delta I_{eq\text{-}sc}$(CCH(I/F))) and the sum of these two currents, corresponding to the maximal CFTR activation ($\Delta I_{eq\text{-}sc}$(max)) ($\mu A/cm^2$). Independent samples t-test, respective p-*values and sample sizes (n) are denoted. Significant p-values (<0.05) are highlighted in bold and with*.*

Parameter 2	Pancreatic function	N	Mean	Standard deviation	p-value
ΔIeq-sc(IF)	PS	3	-10.7100	1.67613	**0.06***
	PI	2	-0.7560	1.41139	
ΔIeq-sc(CCH/IF)	PS	3	-24.2187	11.76715	**0.049***
	PI	2	17.5720	18.30417	
ΔIeq-sc max	PS	3	-34.9287	13.30468	**0.03***
	PI	2	16.8160	16.89278	

When comparing individuals who are PS and PI, we found that organoid swelling in basal conditions of CFTR activation (no treatments) was significantly higher in individuals who are PS, at Fsk concentration of 0.8 μM and 5 μM (Table 4.7).

Table 4.7. Comparison of FIS values in organoids between pwCF who are PS and those who are PI.
Parameter 3 relates to organoid swelling at the different Fsk concentrations tested in the FIS assay (0.02, 0.128, 0.8 and 5 μM) in control conditions (no treatments). Independent samples t-test, respective p-*values and sample sizes (n) are denoted. Significant p-values (<0.05) are highlighted in bold and with*.*

Parameter 3	Pancreatic function	N	Mean	Standard deviation	p-value
FIS 0.02μM	PI	6	78.4759	256.33064	0.840
	PS	4	107.2205	109.66408	
FIS 0.128μM	PI	6	130.4667	342.19125	0.624
	PS	4	228.9556	208.02504	
FIS 0.8μM	PI	6	197.0290	345.21857	**0.009***
	PS	4	1346.9295	718.46899	
FIS 5μM	PI	6	205.7872	412.17765	**0.009***
	PS	4	2062.1819	1270.28082	

Discussion

In this study we have analysed subject-specific CFTR function in rectal biopsies and response to CFTR-modulating drugs in intestinal organoids from pwCF.

Our data indicate that class IV variant p.Arg334Trp is associated with residual CFTR function, which is consistent with an atypical phenotype that these individuals present.[7] Intestinal organoids from the three individuals with the variants p.Arg334Trp showed response to combination therapy with Teza plus Iva in the FIS assay. However, this combination drug is not approved for pwCF with these variants. Only for individual 1, the 17-year-old boy whose diagnosis of CF was made at the age of 15 years and with normal pulmonary function and pancreatic function, a statistically significant response to Iva alone was found, even though it was slightly under the establihed threshold for clinical benefit. However this highlights the importance of personalized medicine, as there is varibility in the response to drugs among pwCF with the same genotype.

For one of these individuals, the 9-years-old girl, with the p.*Phe508del /* p.Arg334Trp genotype with normal pulmonary and pancreatic function and adequate growth (individual 3), triple therapy was also tested which revealed a good response, which may be translated to significant clinical benefit. However, her response to triple therapy was not as good in her organoids as it was for organoids with c.579+1G>T variants nor with p.Phe508del homozygotes, who have a classical CF phenotype.

Class VII variants are misplicing, small indels or large deletions that result in frameshift and total absence of mRNA producing no functional CFTR protein. The classical c.579+1G>T and p.Gln685ThrfsX4 variants are classified in this class, which explains the classical CF phenotype of these individuals.

A new variant, non-existant in the clinical registry CFTR2 (www.CFTR2.org) but recently added to the CFTR Mutation Database (http://www.genet.sickkids.on.ca) was found in individual 7 who had the most severe symptoms of classical CF: c.3321dup. This variant also results in frameshift and thus, no functional protein, and should be classified as class VII. In this case, our data are consistent with the severe classical CF clinical phenotype.

Organoids carrying class VII variants did not show any difference in the FIS assay when incubated with double combination modulator drugs. We can antecipate that CFTR function in these individuals may not be rescued by combination therapy with Iva plus Luma/Teza and, as a consequence, clinical benefit is not expected. For the two individuals (4 and 5) carring the c.579+1G>T variant, the triple therapy was also tested and it revealed a significant response, likely due to the presence of p.Phe508del variant. This response in organoids predict that these individuals will have a significant clinical benefit by taking these triple combination drug. In fact, both these individuals started already triple therapy and we already have a 6-months follow-up clinical assessment in individual 5 which is in accordance with the predicted clinical benefit. We found a SCC and BMI significant improvement (SCC improved from 102mmol/L to 75 mmol/L; BMI z-score improved from -1.81 to -0.46 kg/m^2). However, we have not found a benefit in height and only a modest improvement in pulmonary function (FEV1 was 29% and is now 33.7%) was registered . This highlights the importance of the early start of CFTR modulators in children before irreversible disease sequelae develop. Particularly in the lung, CFTR modulators should be given before bronchiectasis and lung parenchima destruction develop, in contrast to what happened with this individual, who had already significant lung damage. Eventhough weight gain is described in most individuals taking triple therapy, in children if height development has already been compromised their genetic potential for stature may not be achieved [112].

This *ex vivo* study helped us to characterize the rare variant p.Pro5Leu, an N-terminus CFTR cytoplasmic tail missense variant, that has not been previously classified in any functional class, as also having significant residual function [171]. This finding is consistent with a very atypical phenotype that this 2 year-old individual (individual 8) with no clinical manifestations so far and with the clinical description of the mutant in CFTR2 as a "variant with varying consequences, meaning that when combined with another CF-causing variant, may or may not cause CF"[17]. For this individual, results showed that CFTR can be rescued by the Iva/Teza combination therapy. Interestingly, this unclassified variant reinforces the importance of individualized medicine, particularly in rare variants.

Ultimately, we analysed organoids from two individuals homozygous for the most common variant p.Phe508del (individuals 9 and 11). In these individuals we surprisingly found no statistically significant response to combination therapy with Teza/Iva in the the

FIS assay, eventhough this therapy is indicated for individuals with this genotype based on clinical trials [110]. Once again, the importance of personalized medicine is highlighted, as these two particular individuals would probably have no clinical benefit from this combination therapy. On the other hand, the triple therapy which was also tested, revealed a significant response, which will likely translate into significant clinical benefit.

Our data also suggest that CFTR function responses in intestinal organoids may be used to select individuals for treatment with CFTR modulators.

We are aware that drug treatments require a note of caution as it is likely that *in vitro* responses may not be fully reflected *in vivo*, and a specific tissue may present different responses when isolated from its interactive cellular, biologic and social context. However, results from intestinal organoids have been correlated with intestinal voltage measurements and sweat Cl^- concentration in the same individual [16]. It was also shown that mean lung function improvements observed in clinical trials with CFTR modulators in individuals with diferent genotypes, also correlate well with mean FIS response to CFTR modulators in organoids from individuals with the same genotype [16]. By testing efficacy in the individual´s own tissue, organoid testing not only integrates effects in the two CFTR variants present, but also recapitulates any effects that modifier genes and epigenetic factors may have on enhancing/precluding the *in vivo* benefit [16]. Preselecting subjects with uncharacterized CFTR variants for drug therapy using intestinal organoid-based assays has already been reported.[7] Additional proof-of-eficacy could be gained on n-of-1 clinical trials with placebo and active drug treatment chosen in accordance with previous intestinal organoids analysis in order to select the best intervention for each individual, particularly with rare variants [7].

For now, these data suggest that a relatively simple preclinical test may help in clinical decision-making concerning the administration of CFTR modulator therapies in pwCF with rare CFTR variants.

Overall, the relationship between the CFTR genotype and the FIS assay reflects genotype-phenotype relationship in the individuals studied here. Indeed, we found a strong negative correlation between organoid swelling in basal conditions at the Fsk concentrations of 0.8 and 5 μM with the SCT values. Individuals with the higher levels of organoid swelling

(more CFTR function) generally had lower levels of SCT values, which may support the atypical phenotype in these individuals.

We also found higher basal FIS values in intestinal organoids at the Fsk concentrations of 0.8 and 5μM in individuals who are PS, which is in accordance with their atypical phenotype.

At the individual level, specific basal CFTR function FIS measurements in organoids from pwCF allows us to determine whether CFTR residual function occurs and may have value to complement current approaches as a diagnostic and/or prognostic marker for individual pwCF.

In conclusion, data from intestinal organoids ilustrate their value to predict individual responses to approved CFTR modulator therapies, indicating that individuals with p.Arg334Trp or p.Pro5Leu variants may benefit from treatment with combination therapy (Teza/Iva). For individuals who are p.Phe508del homozygotes, or with p.Phe508del/c.579+1G>T and p.Arg334Trp, a greater cilinical benefit would be expected from triple therapy administration.

Chapter 5. Concluding remarks and Future Perspectives

CF management is rapidly changing, and a brighter future is expected for pwCF since CFTR modulator drugs entered clinical practice. During the period of this project new therapies were approved and a wider range of variants were included in the indications for CFTR modulators.

In Chapter 2, our study provided an overview of CFTR modulator drugs approved for paediatric pwCF. Among these individuals from the central region of Portugal some started taking them in one particular period, limiting the number of individuals that we could possibly select for the present study. Of course, these data are always evolving as new individuals are being diagnosed and others are being transferred to the adult centre or unfortunately die before the age of 18 years. Also, clinical characteristics follow their natural evolution in different velocities. For example one 17-year-old pwCF with the p.Phe508del/p.Arg334Trp genotype (individual 1) who was PS when this study started is now PI and taking PERT. A female individual with the p.Phe508del/p.Gln685ThrfsX4 genotype (individual 6) died just a few months after transfer for the adult centre. This highlights the importance of providing available therapies as early as possible before irreversible organ damage occurs.

By clinically and genetically characterizing our pwCF we were able to group them in the different functional classes and establish a genotype-phenotype correlation. We concluded that individuals with variants in classes II and VII (individuals 9,10, 11 and 4, 5, 6 and 7) have a classical phenotypes and that individuals with class IV variants (individuals 1, 2 and 3) have atypical phenotypes. Further in the study, we were able to determine CFTR function for most pwCF in their own tissues, namely in native rectal biopsies, by intestinal voltage measurements and/or by the FIS assay in intestinal organoids. And this proved to be in accordance with clinical findings. Biopsies/organoids from individuals with variants in classes II and VII had no CFTR function, while those from individuals with class IV variants had some CFTR residual function.

Only one individual remained unclassified, i.e., the individual 8 who has the variant p.Pro5Leu (defined as of “varying clinical consequences” in the CFTR2 Database) has an atypical phenotype, even though SCT values (87 mmol/L) were definitely positive. Later in the study we were able to determine the presence of residual function from rectal biopsies using intestinal voltage measurements and the FIS assay, which would probably

classify it has class IV or V variant. Furthermore, we determined this individual may also benefit from therapy with Teza/Iva, even though his genotype is not an indication for this therapy. Unfortunately, we were not able to test triple therapy in this individual's organoids for now.

Another particularly interesting individual was the one with an undescribed variant c.3321dup in one allele and p.Phe508del in the other one (individual 7). The c.3321dup variant was also still unclassified. Clinically, this individual showed a classical phenotype since birth, as he was born with meconium ileus and his SCT values of 120mmol/L confirmed the diagnosis of CF. He is PI and has enteral nutrition support by gastrostomy. He also has severe pulmonary manifestations requiring nocturnal non-invasive ventilation with supplemental oxygen and prolonged admissions to the hospital. Because of genetic analysis, by complete sequencing of the *CFTR* gene by next-generation sequencing, we were able to determine this variant causes a frameshift after Trp1063 and therefore it can be considered a class VII CF-causing variant[174]. To further help in the characterization of this variant, we found that in this individual´s organoids there was no swelling with any Fsk concentration, therefore we concluded that there is no CFTR function, which is accordance with his classical phenotype (Figure 4.11). Consistently, the FIS assay in his organoids revealed no response do tested drugs (Iva and Teza/Iva), thus we do not expect clinical benefit for double therapy with these drugs. However, because he carries p.Phe508del and a zero function variant he is eligible for triple therapy as soon as he completes 12 years of age. However, we did not test it in his organoids.

Before this study the following individuals from our centre were candidates for CFTR modulators (Iva alone, double combination therapies Luma/Iva or Teza/Iva and triple therapy Elexa/Teza/Iva) according to their genotype and age:

-None of the individuals from our centre would benefit from the single therapy with Iva, because none of them has a gating variant.

-All fifteen p.Phe508del homozygous individuals are eligible to take Luma/Iva (since they are all now older than two years [126]) or Teza/Iva as soon as they complete 12 years of age.

- Regarding current INFARMED approval for triple therapy (Elexa/Teza/Iva), 19 out of 23 individuals would be eligible for triple therapy as soon as they complete 12 years of age. Leaving the three individuals with p.Arg334Trp variant and the individual with p.Pro5Leu without any treatment, because these four individuals have some CFTR residual function.

With the present study, and based on the FIS assay of the tests on the 10 individual´s intestinal organoids, first we demonstrated the usefulness of functional assays to determine CFTR function using intestinal voltage measurements and/or the FIS assay for CF diagnosis/prognosis. These assays can be used to determine basal CFTR function in rare variants that may even be new and not fully characterized in international databases (like c.3321dup and p.Pro5Leu). These approaches have allowed us to determine whether there is CFTR residual function or no CFTR function in these individual's own tissues.

Secondly, we found additional indications for combination therapy (Teza/Iva):

- Individuals with p.Phe508del in one allele and the p.Arg334Trp or p.Pro5Leu variants in the other allele may also benefit from combination therapy with Teza/Iva.

- Individuals with the c.579+1G>T, p.Gln685ThrfsX4 and c.3321dup variants are not expected to have any clinical benefit from taking combination therapy (Teza/Iva).

Thirdly, with this study we also demonstrated that individuals with variants that exhibit some residual CFTR function, like p.Arg334Trp are very good responders to the triple therapy. In Portugal, this demonstration is of particular interest, so that INFARMED agrees to provide triple therapy for these individuals.

Fourthly, for the individual with p.Pro5Leu variant (individual 7) there is still no approved treatment to be reimbursed by the NHS. It would be of interest to continue this study using FIS assay to test the triple therapy in this individual and in all individuals carrying rare and uncharacterized variants.

Another interesting finding from the current study is the phenotypic variability that we found among pwCF with the same genotype. Particularly, in individuals homozygous for the p.Phe508del variant, who represent the largest group at our centre, as expected. This raises the question that other genetic, epigenetic, cellular, or biological factors also have significantly influence on the clinical spectrum of the disease. Environmental factors,

such as compliance to therapy or socioeconomic status, are other important contributors to disease course.

In the present study, intestinal voltage measurements and FIS assay were only performed in two individuals with this genotype (intestinal samples from individual 10 were not viable; the remainder 12 individuals homozygous for the p.Phe508del variant did not agree to participate). Anyway, we found a difference in their responses to triple therapy, even though CFTR rescue was significant in intestinal organoids from both of them. It will be interesting to see whether this difference translates into clinical responses too. This results highlights the importance of personalized therapy, even for individuals within the same genetic characteristics.

This study was carried out in children and adolescents with CF, and not in adults. This is important because CF is a progressive disease and it is crucial to determine the best therapeutic option early in life before the pathological lesions become irreversible.

We further characterized pwCF from our centre in terms of intestinal manifestations of CF, as studies on this topic are much scarcer in comparison to the pulmonary involvement. We found an elevated median value for faecal inflammatory marker – calprotectin for pwCF, particularly in individuals who are PI. However, the clinical relevance of this finding is still to be determined as no correlation with nutritional status or gastrointestinal symptoms was found.

Taking advantage of the fact that individuals accepted to perform rectal biopsies for the intestinal voltage measurements and intestinal organoids study, we also used these tissues from 11 individuals for histologically analysis. The only particular alteration found was unspecific focal inflammation present in four individuals which were interestingly associated with higher faecal calprotectin levels (143ug/g;IQR=249 vs 19ug/g;IQR=30; p=0.024). We were also able to determine the sensitivity of calprotectin levels for the presence of histological inflammation which was 100% and specificity which was 86%.

Limitations

We are aware of the limitations of this study as it has a small number of participants (23 individuals of which in 11 rectal biopsies were performed) from only one single paediatric centre. However, the main aim of the study was to characterize and determine among children and adolescent with CF followed-up at our centre which ones are candidates for new CFTR modulator drugs by determining the potential benefit of these modulator drugs in their own tissues.

We are also aware that drug treatments require a note of caution as it is possible that *in vitro* responses may not be fully translated *in vivo*. However, other authors have shown that results from intestinal organoids correlate with intestinal voltage measurements and SCT values in the same individual [16]. It was also shown that mean lung function improvements observed in clinical trials with CFTR modulators in individuals with different genotypes, correlate well with mean FIS response to CFTR modulators in organoids from individuals with the same genotype [175]. Preselecting subjects with uncharacterized CFTR variants for drug therapy using rectal organoid-based assays has already been reported [176].

Future perspectives

Although the combination of high throughput screening of small molecule libraries and medicinal chemistry have resulted in amazing new effective modulator therapies for most pwCF, unfortunately, there are still ~20% of pwCF that still cannot benefit from available therapies because their rare variants are not listed in approved indications[51].

Moreover, it was estimated that worldwide only 12% of eligible individuals for these drugs are actually taking them [177], due to their high cost [177]. This figures take into account pwCF from low and middle-income countries where these therapies are not also available for economic reasons or where the diagnosis (genetic or not) of CF is not confirmed [178].

Many CF-causing variants among the 2,100 identified to date occur in a very small number of individuals worldwide, and thus large randomized trials for each rare variant is implausible. A EU-funded project called "HIT-CF-Human Individualized Treatment for CF" (www.hitcf.org) is ongoing in 16 European different countries, including Portugal. The goal of the project is to get novel modulator drugs to pwCF who carry (ultra-) rare variants by predicting their clinical response to these novel drugs using the

FIS assay in intestinal organoids, i.e., the same assay used in the present study, but at high-throughput. Additional proof-of-efficacy could be gained on n-of-1 clinical trials with placebo and active drug treatment alternating in the same individual (with adequate washout periods), with individuals selected in accordance with previous intestinal organoids analysis in order to select the best therapy for each individual [19].

With the emergence of HEMT, it is essential to re-evaluate the need to maintain the burden of chronic symptomatic treatments, like daily airway clearance techniques and inhaled mucolytic and antibiotics. In 2020, a randomized clinical trial was conducted to determine whether nebulized hypertonic saline or dornase alfa could be safely withdrawn from the daily treatment regime of pwCF on modulators [179]. Conclusion was that in individuals with cystic fibrosis on HEMT with relatively well preserved pulmonary function, discontinuing daily hypertonic saline or dornase alfa for 6 weeks did not result in clinically meaningful differences in pulmonary function when compared with continuing treatment [180]. For dietary advice and antibiotic use it is also crucial to answer this question.

Initiation of CFTR modulators as early ages as possible may prevent worsening of CF condition as, for example, pancreatic exocrine failure. Thus, timely CF detection through NBS and early establishment of a CF diagnosis is crucial [104]. Furthermore, in a ferret model of p.Gly551Asp variant CF, *in utero* administration of Iva reduced the rate of meconium ileus and protected the male reproductive tract [181]. These data both emphasize the importance of normal CFTR function *in utero* and demonstrate that earlier modulator administration, even during pregnancy, is optimal. For example, usage of CFTR modulator drugs during pregnancy should be considered subsequently to a positive prenatal CF diagnosis, if pregnancy use of modulators is proven to be safe [181].

The positive clinical results so far obtained from the CFTR modulator drugs have encouraged competitive research in this field. Accordingly, several pharma companies are developing novel drugs and drug combinations on what is called the Cystic Fibrosis drug developing pipeline (https://www.cff.org/Trials/Pipeline) [4]. Thus, more progress is expected in the near future from these developments.

Read-through agents are molecules which allow full-length protein translation in individuals with premature stop variants (Class I variants), that would otherwise lead to truncated (non-functional) proteins [4]. Recently, a clinical trial conducted with ELX-02, a read-through compound, with ivacaftor did not achieve statistical significance for efficacy endpoints in Phase 2 study in Class I CFTR variants.

Symptomatic therapies for the management of CF are also continuing to be updated, particularly because of the emerging threat of antimicrobial resistance, with new antibiotics and other antimicrobials targeting biofilm formation, bacterial virulence and epithelial damage [4]. Trials of new agents and formulations to attenuate inflammation and/or promote its resolution without blocking the critical inflammatory response and developments of innovative mucolytic agents are also underway [4].

Notwithstanding, new complications are emerging in adult pwCF, as they live longer. Obesity, diabetes, dyslipidaemia and also gastrointestinal cancer and lymphomas are becoming more frequent and are leading to the development of new screening guidelines [51].

Nevertheless, CFTR modulator drugs are likely to be the most important development in CF care for a generation, and possibly ever. For a disease that used to be universally fatal in childhood, this is one of the best success stories of modern medicine, as they offer hope and a bright new future to the majority of pwCF and their families.

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