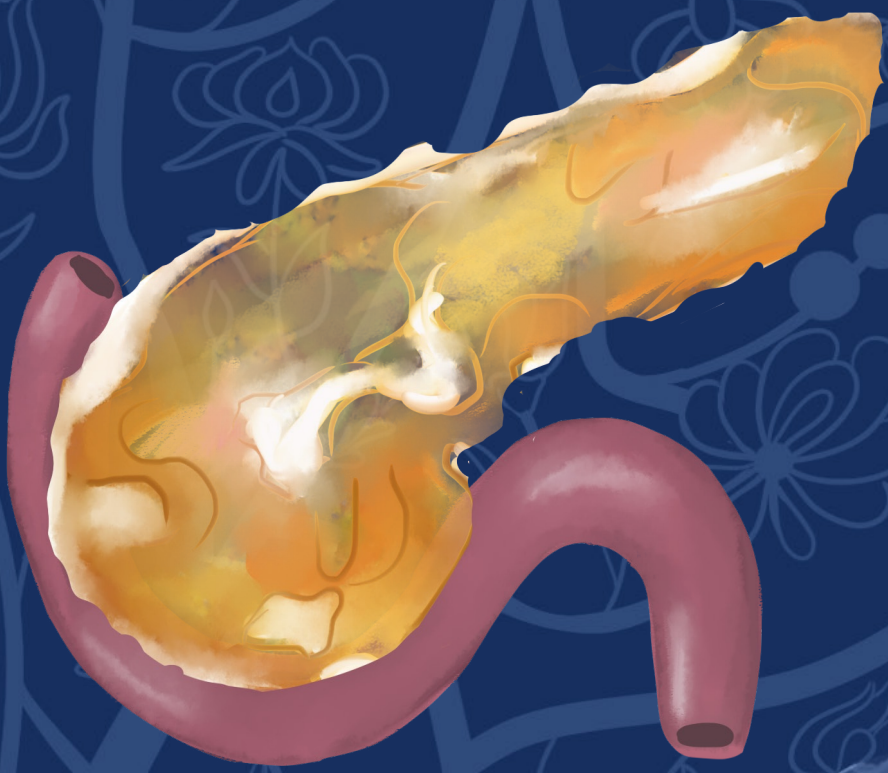
CFTR Function in Pancreatitis



Dóra Angyal

Propositions

accompanying the thesis

CFTR Function in Pancreatitis

Dóra Angyal

1. Impaired CFTR-mediated bicarbonate transport is a key pathogenic factor in both cystic fibrosis and CFTR-related pancreatitis (this thesis).
2. Pro-inflammatory cytokines can stimulate CFTR-dependent anion secretion in pancreatic ductal cells, potentially modulating disease course (this thesis).
3. Assessment of CFTR function relevant to CFTR-related pancreatic disease using the currently available biomarkers of CFTR dysfunction is challenging (this thesis).
4. Expanding the use of CFTR modulators beyond cystic fibrosis requires in vitro evaluation of their impact on ion transport in the individual genetic context, and possibly tissue, of patients affected by CFTR-related disease (this thesis).
5. Patient-derived pancreatic ductal organoids are a promising platform for personalized medicine in CFTR-related pancreatitis (this thesis).
6. As genetic testing becomes more readily available, CFTR-related diseases are diagnosed with increasing frequency.
7. The diagnostic process of CFTR-related disease is a complex maze.
8. The therapeutic benefit of medications can extend beyond their originally intended indications and remain only partially understood.
9. Electrophysiology remains relevant for understanding biological systems, despite the rise of fluorescent tracers and omics technologies.
10. As conclusions are embedded in a specific context, caution is warranted in their interpretation, particularly in an era where the attention span of scientists and the public is increasingly reduced to the brevity of clickbait headlines.
11. A comfort zone is a beautiful place, but nothing ever grows there.

CFTR Function in Pancreatitis

Dóra Angyal

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Dóra Angyal

born in Budapest, Hungary.

Erasmus University Rotterdam



Doctoral committee

Promoters:	Prof.dr. M.P. Peppelenbosch
	Prof.dr. M.J. Bruno
Co-promoters:	Dr. M.J.C. Bijvelds
	Dr. H.R. de Jonge †
Assessment committee:	Prof.dr. C.K. van der Ent
	Prof.dr. J.C. Escher
	Dr. G.M. Fuhler

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Chapter 1

General introduction

The recent development of cystic fibrosis transmembrane conductance regulator (CFTR) modulators has drastically improved the health outcomes of many people with cystic fibrosis (CF) (pwCF). The application of modulators is now restricted to pwCF carrying a limited selection of *CFTR* mutations. However, CFTR modulators may benefit a wider range of subjects, and, conceivably, may also be applied for treating other diseases associated with impaired CFTR function, such as pancreatitis. An increased understanding of the role of CFTR in bicarbonate secretion in pancreatic ductal cells, whose scientific investigation and understanding lags behind that of acinar cells, could aid development of targeted drugs for pancreatitis. In this thesis we present personalized medicine approaches, novel disease models, and advances in endoscopic procedures to assess the response to CFTR modulators of rare CF-causing *CFTR* mutations, characterize *CFTR* mutations associated with pancreatitis, and develop models to study CFTR-dependent anion transport in pancreatitis.

Cystic fibrosis (CF)

CF is one of the more common potentially fatal inherited diseases, estimated to affect >90,000 people worldwide (1). Over 50,000 people in Europe and >1,600 people in the Netherlands live with CF (2, 3). As the prevalence of CF in regions without well-established registries is probably underestimated, global prevalence is likely even higher than the current estimate (1, 4). The prevalence of CF is also rising, because improved clinical management and CFTR targeting therapies have increased life expectancy in countries where this care is available (2, 4, 5). Moreover, inclusion of CF in newborn screening programs and advances in genetic testing have increased the number of individuals that are diagnosed (4, 6).

CF was first recognized as a unique disease entity based on mucus plugging of the pancreatic ducts in infants with celiac disease in 1938 (7). About fifteen years later, it was discovered that high salt concentrations in sweat are typical of CF (8), and a diagnostic sweat test was developed (9). Impaired epithelial chloride transport was identified as the underlying defect in the sweat ducts (10). Using positional cloning, the CF causing gene, *CFTR*, was discovered in 1989, and it was demonstrated that this gene encodes an integral membrane protein that allows the flow of anions across the plasma membrane of (mostly) epithelial cells in CF-affected tissues (11-13).

Currently, CF is recognized as an autosomal recessive multi-organ disease caused by loss-of-function mutations in *CFTR* (14). CFTR is expressed in multiple tissues, including the airways, sweat glands, intestine, liver, pancreas and male reproductive tract. In these epithelial tissues, CFTR has a key role in the regulation of luminal fluid levels and the viscosity of secreted mucus (15-18). When CFTR function is impaired, CF-related luminal dehydration and mucus plugging is thought to lead to diverse secondary disease manifestations, including chronic sinusitis, lower respiratory tract

infections and inflammation with progressive lung damage, intestinal obstruction, liver disease, pancreatic insufficiency, malnutrition and male infertility (4, 14).

Cystic fibrosis transmembrane conductance regulator (CFTR)

Epithelia form the physical barrier between the body and its environment. Among other functions, epithelial cells regulate electrolyte and fluid homeostasis via the activities of various solute and ion transport mechanisms (19).

CFTR mediates anion transport across the apical surface of several types of epithelia (20). CFTR is a phosphorylation-regulated chloride and bicarbonate channel, displaying adenosine triphosphate (ATP)-dependent gating. CFTR is the only ion channel in the family of ATP-binding cassette (ABC) transporters (21). CFTR comprises of two transmembrane domains (TMD1 and TMD2) which interact to form a single anion selective pore, two cytoplasmic nucleotide-binding domains (NBD1 and NBD2), which bind and hydrolyze ATP to regulate channel gating (22, 23), and a regulatory (R) domain which contains multiple phosphorylation sites (21, 24) (Figure 1). Phosphorylation of the R domain, typically catalyzed by the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), disengages it from its inhibitory position, allowing dimerization of the NBD domains and opening of the channel pore (25, 26).

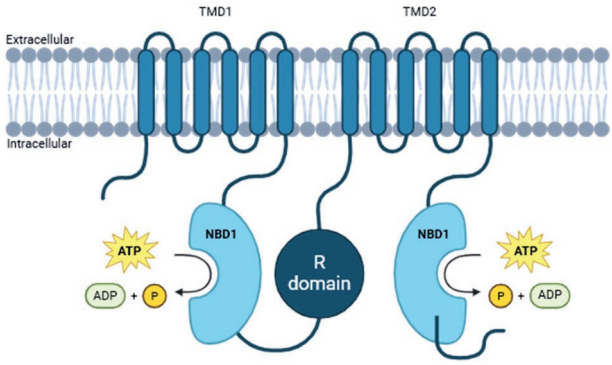


Figure 1. Schematic model of CFTR structure. ATP/ADP, adenosine tri-/-diphosphate; P, phosphate; TMD, transmembrane domain; NBD, nucleotide binding domain; R domain, regulatory domain. Adapted from Sheppard and Welsh 1999 (24). Created with BioRender.com.

In most CFTR expressing epithelia, with the notable exception of the sweat ducts and, potentially, alveoli (20), activation of CFTR-mediated chloride and bicarbonate secretion to the lumen, creates an electrical potential difference across the epithelium that drives paracellular sodium secretion (19) (Figure 2). In turn, net sodium and chloride secretion provides the driving force for osmotic water movement. CFTR can mediate bicarbonate transport directly, but CFTR-mediated chloride efflux can also

serve to drive bicarbonate secretion via separate ion transport mechanisms, that is chloride/bicarbonate exchangers, such as AE2 (*SLC4A2*) in epithelial cells of the biliary tract, and DRA (*SLC26A3*) and PAT1 (*SLC26A6*) in epithelia of the intestines, the pancreatic ductal tree and the airways (27-29).

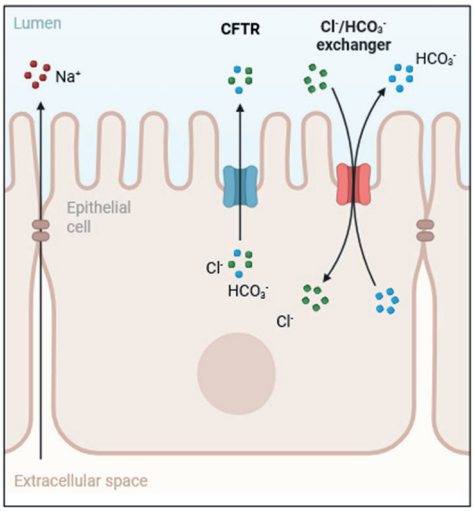


Figure 2. CFTR in epithelial ion transport. CFTR-mediated chloride (Cl^-) and bicarbonate (HCO_3^-) secretion to the lumen, drives paracellular sodium (Na^+) secretion. CFTR-mediated chloride secretion can drive bicarbonate secretion via chloride/bicarbonate exchangers. Created with BioRender.com

Defective CFTR-dependent chloride and bicarbonate secretion not only perturbs epithelial fluid homeostasis, but also, by reducing the secretion of base equivalents (bicarbonate), impairs luminal pH regulation (30). In affected epithelial tissues, the lowering of the luminal pH impairs unfolding of a group of secreted glycoproteins, mucins, that are essential for mucus formation, leading to the production of an abnormally viscous mucus layer (15-18, 31, 32). In the airways, this accretion of mucopurulent secretions impairs mucociliary clearance of pathogens, leading to chronic infection, inflammation and, ultimately, progressive lung damage (33). Dysregulation of the luminal pH is thought to also impair the host defense response to pathogens in the airways (34). Further, in the intestinal tract, increased mucus viscosity may contribute to malabsorption of nutrients and cause obstruction (35). In the pancreas, a low luminal pH may contribute to premature activation of digestive enzymes, in turn, triggering tissue damage and inflammation (36). Finally, in the biliary tract, impaired pH regulation may increase bile acid cytotoxicity, which, combined with the accretion of hyperviscous mucus, may contribute to the development of CF-related liver disease (CFLD) (37).

Other than in most other CFTR-expressing tissues, in the sweat glands, CFTR participates in electrolyte absorption (38). Consequently, in pwCF, because CFTR-mediated chloride uptake from the lumen of the sweat ducts is reduced, sweat chloride concentrations are elevated (8). Thus, measurement of sweat chloride concentration, as performed in the Gibson-Cooke test, can serve as a diagnostic tool, and levels >60 mmol/L are considered pathognomonic for CF (9). Other routinely applied diagnostic tools to monitor CFTR function are nasal potential difference (NPD) measurement (*in vivo*) (39-41) and intestinal current measurement (ICM) in rectal biopsies (*ex vivo*) (42).

To date, more than 2000 mutations in *CFTR* have been identified (43), and, of those annotated, more than 1000 are considered CF-causing (44). Traditionally, *CFTR* mutations are categorized into six classes based on defects in protein synthesis and channel function (45, 46):

I. No production of full-length protein
II. Production of misfolded protein, leading to premature degradation and impaired trafficking to the cell surface
III. Defective gating of CFTR channel
IV. Decreased conductance of CFTR ion pore
V. Reduced synthesis of CFTR protein
VI. Decreased stability of CFTR protein at the cell surface

Class I-III mutations have no or little residual function, and are associated with a more severe phenotype with pancreatic insufficiency (PI) at birth, while class IV-VI mutations may retain some function, often associated with pancreatic sufficiency (PS) (4, 47).

CFTR modulators and other CF therapies

Up until quite recently, management of CF relied on symptomatic treatment strategies, which were principally directed at preserving lung function and improving the nutritional status of pwCF. This includes the use of mucolytics, antibiotics, physiotherapy, high calorie diets, and pancreatic enzyme replacement therapy (14). Aided by an ever progressing understanding of the underlying molecular defect, the past four decades have seen substantial improvements in the prognosis of pwCF. Whereas before an often fatal disease of infancy, half the pwCF born in North America today are predicted to live beyond 61 years (48).

Improved understanding of the molecular defect in CF has, among other developments, led to the recent introduction of a novel class of therapeutics, so-called CFTR modulators, which are small molecule compounds that aim to restore the function of the mutant *CFTR* product. There are two distinct types of modulators: (1) potentiators improve CFTR channel gating and/or conductance, whereas (2) correctors improve (co-translational) folding and subsequent trafficking of CFTR protein to the plasma

membrane (49). Ivacaftor (IVA, VX770) is a potentiator that stabilizes the open state of phosphorylated CFTR, thereby improving channel gating (50-52). In Europe, IVA monotherapy is approved for nine class III and the *R117H* mutations (53). The combination of IVA with lumacaftor (LUM, VX809), a corrector which improves trafficking of some CFTR variants (54), is approved for pwCF homozygous for the *F508del* mutation (55). The combination of IVA with the corrector tezacaftor (TEZ, VX661) showed similar efficacy as the IVA/LUM combination, but with less side-effects (56, 57). The combination of IVA and TEZ with elexacaftor (ELX, VX445; together abbreviated as ETI) resulted in a significant improvement in clinical outcome in pwCF carrying at least one *F508del* allele (58, 59). First licensed in 2019, it is now approved for this cohort (60), which in regions where the incidence of CF is high, represents >85% of the CF population (48).

Other therapies targeting the underlying defect in CFTR have not yet reached patients (14, 49). The latest progress in this field includes the development of amplifiers, which increase the amount of mRNA encoding mutant CFTR. Treatment with amplifiers, by increasing the production of mutant mRNA and protein, is projected to enhance the efficacy of treatment with modulators when combined. Further, read-through agents may permit the production of full-length protein from transcripts containing premature stop codons. However, these have not shown clinical efficacy yet. Experimental therapies directed at introducing wild type CFTR encoding DNA or mRNA in CF-affected cells have been the focus of intense study, but yet await clinical application.

CFTR-RD and CFSPID

The term CFTR-related disorder (CFTR-RD) is used to designate a range of disorders that are associated with CFTR dysfunction, but do not fulfill diagnostic criteria for CF (61) (Figure 3). Publication of a unifying definition of CFTR-RD nearly fifteen years ago increased awareness of CFTR-RD among non-CF specialists, leading to a marked rise in the number of detected cases. The varied clinical presentation of CFTR-RD has highlighted the complex relation between the basic defect and disease presentation (62). CFTR variants associated with CFTR-RD typically display residual channel function, and, unlike in CF, disease manifestations in CFTR-RD are often mild and/or limited to a single organ (62). The widespread implementation of genetic screening procedures has underlined the association between various CFTR variants and diseases such as pancreatitis, congenital bilateral absence of the vas deference (CBAVD) and disseminated bronchiectasis (62, 63).

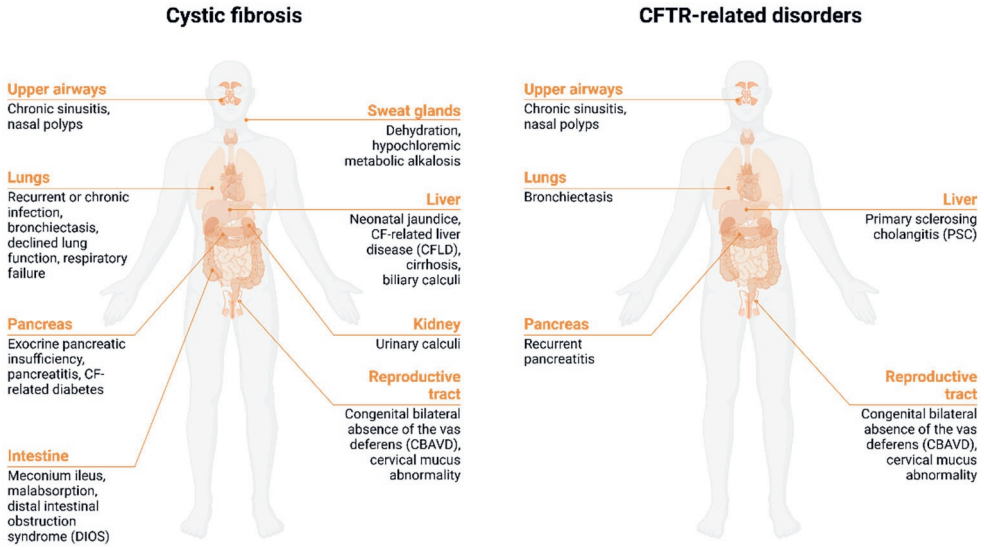


Figure 3. Clinical manifestations of CF and CFTR-RD. Adapted from Shteinberg et al. 2021 (14). Created with BioRender.com

The implementation of newborn screening strategies, aimed at early diagnosis to allow early intervention to minimize irreversible damage and prevent malnutrition, has led to an increase in the detection of individuals with *CFTR* variants who are apparently healthy. This group is termed CF screen-positive, inconclusive diagnosis (CFSPID) in Europe (64) and CFTR-related metabolic syndrome (CRMS) in North America (65). Individuals in this category may still develop clinically significant symptoms. Because the level of (residual) function of such CFTR variants can vary widely between individuals and, possibly, even between tissues, the clinical presentation of CFSPID/CRMS is highly variable, and comprises a broad spectrum of symptoms (4, 66) (Figure 4). As symptoms may present later in life, the burden and benefits of follow-up should be carefully weighed. The development of consensus guidelines for management of CFSPID/CRMS newborns is ongoing (67, 68).

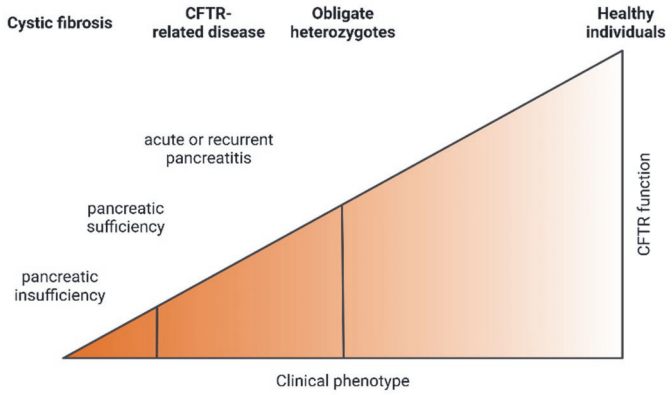


Figure 4. CFTR function and clinical phenotype. Adapted from Bell et al. 2020 (4). Created with BioRender.com.

The relation between genotype and phenotype

Over the last decades, as exome sequencing is increasingly used for diagnostic purposes, the number of variants with uncertain clinical consequences identified has risen substantially (62). Such patient data has been collected in publicly accessible databases, e.g. CFTR2 (44) and CFTR-France (69, 70), which offer a wealth of information on the relation between genotype and phenotype, and can be used to estimate the disease liability of individual *CFTR* variants. Subjects carrying *CFTR* variants of varying clinical consequence (VVCC) may display a range of clinical manifestations. Typical of VVCC carriers, even the phenotype of those carrying identical variants may vary considerably (71). Also, the clinical response to modulator treatment may vary between those carrying identical variants (58, 59). These observations indicate that the function of CFTR is influenced by factors other than the *CFTR* genotype. These include modifier genes (72, 73), whose effects on CFTR activity can also vary between different tissues (74).

As these are mostly rare variants, carried by only few individuals, it is impractical to assess their response to CFTR modulator drugs in classical clinical trials. Therefore, because of their low prevalence and the inherent difficulties in the design of clinical trials, other methods for evaluating drug responses are indicated. For instance, FDA-approval for use of IVA for some rare CFTR mutations was based on an electrophysiological assay performed in a heterologous cell expression system (FRT cells) (75). Another option is the use of patient-derived cells, stored in *living* biobanks, which can be used for bioassays that test the effect of modulator therapy on mutant CFTR function *in vitro* (theratyping).

The role of CFTR dysfunction in pancreatitis

Pancreatitis has a high socioeconomic burden (76) and is a widely supported focus of research (77). Pancreatitis is a multifactorial, complex inflammatory disorder of the pancreas that develops through an interaction between genetic factors and multiple environmental and metabolic triggers. Inflammation is thought to be initiated by the premature conversion of the zymogen trypsinogen into the active protease. This inappropriate activation of trypsin within acini and the ductal tree triggers tissue injury and inflammation. Mutation of one of several genes thought to be involved in the intra-pancreatic activation of trypsin (IPAT) has been shown to confer an increased risk of pancreatitis. One such gene is *CFTR*, and this set further includes *PRSS1*, *SPINK1*, *CTRC*, *CTRB1*, *CTRB2* (trypsin-dependent pathway), *CPA1*, *CEL* (protein misfolding pathway), and *CLDN2* and *CASR* (ductal pathway) (36). Thus, the association between CFTR dysfunction and pancreatitis is not only evident in CF and CFTR-RD, but also detected in idiopathic and hereditary forms of pancreatitis (78). It has been estimated that between 20-45% of idiopathic cases carry mutations in *CFTR* on one or two alleles (79-84).

Among the *CFTR* mutations associated with pancreatitis, rhinosinusitis, and male infertility, but not CF, nine variants showed normal chloride, but reduced bicarbonate secretion in a heterologous CFTR expression model (R74Q, R75Q, R117H, R170H, L967S, L997F, D1152H, S1235R, and D1270N) (79, 85). The identification of these CFTR-BD (bicarbonate defective) variants appears to underline the importance of CFTR in the postprandial production of a highly alkaline pancreatic juice (86). Indeed, both computational modeling and experimental evidence from animal studies indicate that CFTR is crucial for pancreatic ductal bicarbonate secretion (87, 88).

Presently, it is largely unclear whether CFTR modulators restore bicarbonate transport in CFTR variants associated with either CF and/or a CFTR-RD, because most bioassays used for preclinical testing of modulators assess CFTR-mediated chloride secretion, or attendant fluid transport, but not bicarbonate transport (89).

Acquired CFTR dysfunction in pancreatitis

Common causes of non-hereditary forms of pancreatitis are exposure of the ductal epithelium to environmental and metabolic triggers, namely bile acids (typically resulting from obstruction of the papilla of Vater by gall stones), tobacco smoking, alcohol abuse, medical procedures and associated trauma, autoimmune disease (auto immune pancreatitis) and drug toxicity (90). Some of these forms have been linked to an acquired dysfunction of (wild type) CFTR. For instance, exposure to ethanol and fatty acids was proposed to lower CFTR localization in the plasma membrane, and to reduce fluid secretion in pancreatic ductal epithelium (91). The immunosuppressive drug azathioprine, a thiopurine known to increase the risk of pancreatitis, was also reported

to reduce CFTR localization in the plasma membrane, and to reduce ductal anion and fluid secretion (92). In a mouse model of auto immune pancreatitis, treatment with CFTR modulators (C18; IVA) reduced inflammation and tissue damage, and partially rescued ductal fluid secretion (93). Together, these findings suggest that strategies to restore and/or enhance CFTR function in the pancreatic ductal epithelium may be used to treat pancreatitis, and/or prevent recurrence.

The use of organoids to study CFTR pathophysiology and evaluate therapeutic interventions

Organoids are self-renewing cell cultures which maintain key characteristics of their tissue of origin (94). Organoids are generated from the stem cells contained in tissue specimens, such as (intestinal) biopsies (95, 96) (Figure 5A). Patient-derived organoids, stored in living biobanks, can subsequently be used to study the pathophysiology of CFTR-RD (97), and to predict the clinical effects of CFTR modulators (98).

One assay that is used to monitor CFTR function is the forskolin-induced swelling (FIS) assay (99) (Figure 5B). In intestinal organoids, CFTR mediates anion secretion into the enclosed lumen, which, in turn, triggers osmotic fluid transport. The CFTR-dependent accumulation of fluid causes the organoids to swell, which can be monitored using microscopic techniques. Based on this principle, intestinal organoids can be used to evaluate (1) the impact of different *CFTR* mutations on channel activity, and (2) the effect of modulators on the function of specific CFTR variants. However, organoid swelling is highly dependent on chloride transport, and, therefore, FIS is less suitable to study bicarbonate transport (100).

Organoid-derived cells can be used to culture epithelial monolayers on a permeable substrate (filter) (Figure 5C). As CFTR-mediated anion secretion causes a change in the transepithelial electrical potential difference, electrophysiological measurements on such organoid-derived epithelial monolayers can be used to assess (mutant) CFTR function (Figure 5D), analogous to the use of intestinal biopsies in ICM and the FRT cell-based assay. This technique provides access to both the apical and basolateral compartments of the epithelium, allows separate measurement of chloride and bicarbonate transport, and can be used to evaluate the effect of CFTR modulators (101). Whereas well-suited to detect (rescue of function at) low levels of CFTR activity, FIS tends to underestimate the activity of more moderately affected CFTR variants. In comparison, electrophysiological assays have an extended dynamic range, rendering them more suited to evaluate CFTR variants with a comparatively high residual activity.

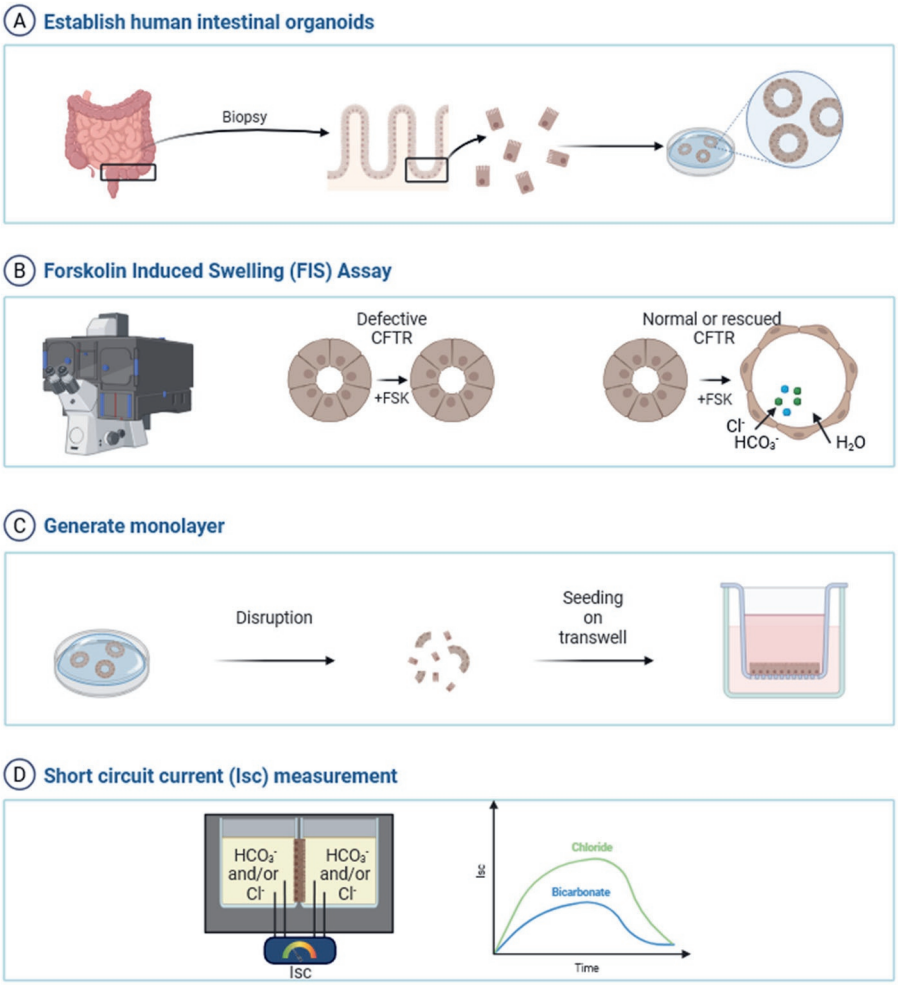


Figure 5. Patient-derived organoids as models to study CFTR-dependent fluid and ion transport. (A) Intestinal organoids are generated from stem cells obtained from tissue biopsies. (B) Forskolin-induced swelling (FIS) assay measures forskolin induced, CFTR-dependent fluid secretion and organoid swelling. Swelling is reduced/absent in organoids derived from pwCF. The assay can be used to evaluate the response to CFTR modulators. (C) Organoid-derived epithelial cells can be cultured as monolayers on permeable supports. (D) Electrophysiological measurements in these monolayers enable assessment of CFTR-mediated chloride and bicarbonate transport and evaluation of CFTR modulator responses. Created with BioRender.com

Aim and outline of this thesis

This thesis addresses the role of impaired CFTR-dependent bicarbonate transport in CF and one of the CFTR-RD, pancreatitis. The principle aim was to characterize CFTR-mediated bicarbonate transport in near-native epithelium, to investigate the role of CFTR (dys-) function in the etiology of pancreatitis and the effect of CFTR modulator therapy on bicarbonate transport.

In **chapter 2**, current knowledge of CFTR-dependent bicarbonate transport in pancreatic ductal cells, and its role in the development of CF-related pancreatic disease and in other (CFTR-related and acquired) forms of pancreatitis is reviewed. Furthermore, we discuss the perspectives for CFTR modulator therapy in the treatment of exocrine pancreatic insufficiency and pancreatitis.

In Part I, CFTR-mediated bicarbonate and chloride secretion in individuals carrying *CFTR* mutations diagnosed with CF, CFTR-RD or pancreatitis is investigated.

In **chapter 3**, the response to the CFTR modulator combination elexacaftor/tezacaftor/ivacaftor (ETI) of four rare CFTR variants, at present not eligible for modulator therapy, in patient-derived intestinal organoids and epithelial monolayers is evaluated.

In **chapter 4**, bicarbonate and chloride transport mediated by the pancreatitis-associated CFTR-BD variant D1152H and the effect of ivacaftor thereupon is assessed in intestinal organoid-derived epithelial monolayers.

In **chapter 5**, various clinical, *ex vivo* an *in vitro* techniques are used to investigate CFTR-dependent chloride and bicarbonate transport, and the putative beneficial effects of modulator therapy, in a panel of 32 pancreatitis patients carrying rare *CFTR* mutations.

In Part II, CFTR function in pancreatic ductal models is studied.

In **chapter 6**, a pancreatic ductal organoid model is presented, and the effect of pro-inflammatory cytokines on CFTR-dependent ductal anion secretion is investigated.

In **chapter 7**, the effects of the pancreatitis-associated fatty acids and ethanol on CFTR-mediated anion transport in healthy pancreatic organoids is investigated.

In **chapter 8**, advanced endoscopic techniques are employed to establish pancreatic ductal organoids from chronic pancreatitis patients, and it is demonstrated that these cultures can be used to study the pathophysiology of pancreatitis and for theratyping.

In **chapter 9**, the conclusions, the strengths and limitations, and the future perspectives of our studies are discussed.

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Chapter 2

Bicarbonate transport in cystic fibrosis and pancreatitis

Dora Angyal, Marcel J.C. Bijvelds, Marco J. Bruno, Maikel P. Peppelenbosch, Hugo R. de Jonge

Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

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Abstract

CFTR, the cystic fibrosis (CF) gene-encoded epithelial anion channel, has a prominent role in driving chloride, bicarbonate, and fluid secretion in the ductal cells of the exocrine pancreas. Whereas severe mutations in *CFTR* cause fibrosis of the pancreas *in utero*, CFTR mutants with residual function, or CFTR variants with a normal chloride but defective bicarbonate permeability (CFTR-BD) are associated with an enhanced risk of pancreatitis. Recent studies indicate that CFTR function is not only compromised in genetic but also in selected patients with an acquired form of pancreatitis induced by alcohol, bile salts or smoking. In this review we summarize recent insights into the mechanism and regulation of CFTR-mediated and modulated bicarbonate secretion in the pancreatic duct, including the role of the osmotic stress/chloride sensor WNK1 and the scaffolding protein IRBIT, and current knowledge about the role of CFTR in genetic and acquired forms of pancreatitis. Furthermore, we discuss the perspectives for CFTR modulator therapy in treatment of exocrine pancreatic insufficiency and pancreatitis, and introduce pancreatic organoids as a promising model system to study CFTR function in the human pancreas, its role in the pathology of pancreatitis, and its sensitivity to CFTR modulators on a personalized basis.

Keywords: CFTR, cystic fibrosis, pancreatitis, bicarbonate

1. Introduction

Cystic fibrosis (CF) is a potentially fatal multi-organ disease caused by mutation of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. It is estimated that currently CF affects >100,000 people worldwide, and that by 2025 the number of patients will have increased with 50% (1, 2). *CFTR* encodes a phosphorylation-regulated, adenosine triphosphate (ATP)-gated, epithelial anion channel that mediates chloride (Cl^-) and bicarbonate (HCO_3^-) transport across epithelia, principally located in the respiratory, gastrointestinal and male reproductive tract. The term cystic fibrosis refers to the CF-typical fibrotic lesions in the pancreas, first described in the 1930s (3). In approximately 85% of CF patients fibrosis of the pancreas starts *in utero*, progressing to a complete loss of exocrine pancreas function soon after birth, i.e. exocrine pancreatic insufficiency (4). The severely reduced release of HCO_3^- and digestive enzymes into the upper intestinal tract impairs neutralization of gastric acid and causes malabsorption of nutrients.

Pancreatitis is a complex inflammatory disease of the acinar and ductal epithelium, which probably results from premature activation of digestive enzymes (5). Currently, there is no specific therapy available, and treatment relies on supportive care. Pancreatitis is one of the three most common causes of gastrointestinal disease-related hospitalizations, and is associated with high morbidity and mortality (6). Acute pancreatitis (AP), recurrent AP (RAP) and chronic pancreatitis (CP) are thought to represent a disease continuum (7). As opposed to the monogenic disease CF, in pancreatitis, multiple (epi-)genetic, metabolic and environmental factors combine, and lead to a complex pathology (8, 9). A substantial body of evidence indicates that the loss of ductal CFTR-dependent fluid and HCO_3^- secretion may precipitate the development of pancreatitis (10). This is perhaps most poignantly illustrated by the fact that pancreas sufficient CF patients, i.e. those who have retained some level of exocrine pancreatic function, are at increased risk of developing (R-)AP and CP (11). Furthermore, it has been shown that carriers of a CF allele are at increased risk of developing pancreatitis (12).

CFTR-mediated HCO_3^- secretion not only drives osmotic fluid secretion in ductal structures, but is also required for controlling the pH at the epithelial surface, and the proper expansion of secreted mucins. Thus, in CF, a lowering of the luminal pH is thought to result in the accumulation of a hyper-viscid mucus that blocks the ductal structures, and promotes microbial colonization and inflammation. In addition, in the pancreas, high levels of HCO_3^- are thought to maintain secreted digestive enzymes in an inactive state, while still located in the ductal tree (13).

In view of the apparent relevance of HCO_3^- in the pathophysiology of CF and CFTR-related disorders (CFTR-RD) such as pancreatitis, it is perhaps surprising that most

research into the function of CFTR has focused on its role as a Cl^- channel (14). Perhaps due to the fact that it is readily formed from and converted to CO_2 and H_2O, accurate assessment of HCO_3^- concentration and transport is challenging, which may explain why CF biomarkers and therapy testing are Cl^- biased (15). This is true for clinical nasal potential difference (NPD) and sweat Cl^- tests which are Cl^- based, as well as CFTR modulator testing which are based on iodide quenching or membrane potential measurements, while intestinal current measurements (ICMs) and Forskolin-Induced Swelling (FIS) assays measure combined Cl^- and HCO_3^- transport.

This review summarizes our current knowledge of the role of CFTR in pancreatic ductal fluid and HCO_3^- transport, and the role of CFTR dysfunction in pancreatitis. As is outlined in the next section, CFTR is a critical element of the ion transport machinery in ductal epithelial cells, which, remarkably, is able to accumulate HCO_3^- in human pancreatic juice to levels of up to 140 mmol/L. Several seminal studies published in the last decade point towards a prominent role of impaired CFTR-mediated HCO_3^- transport in the pathophysiology of pancreatitis (16, 17). This offers the encouraging prospect that CFTR modulators, originally developed for treatment of CF, may also be of potential benefit for other pancreas-related diseases.

2. Bicarbonate transport in the exocrine pancreas

2.1 CFTR is indispensable for the accumulation of bicarbonate in pancreatic juice

In the exocrine pancreas, acinar cells secrete various digestive enzymes in a small volume of isotonic, NaCl and H^+-rich fluid, after which the ductal epithelium serves to exchange the secreted Cl^- for HCO_3^-, to produce an alkaline fluid (pH 8.0–8.5) (13, 18, 19). It is estimated that the human exocrine pancreas secretes up to 1 L of pancreatic juice per day (18, 20, 21). Apart from delivering digestive enzymes to the proximal small intestine, making it essential for digestion of (macro-)nutrients, pancreatic juice serves to supply base equivalents for neutralization of the gastric acid entering the small intestine from the stomach. In humans, the HCO_3^- concentration in pancreatic juice can reach levels of 140 mmol/L (22, 23). Similarly high levels of HCO_3^- were observed in pancreatic juice of guinea pigs, whereas, in contrast, in mouse and rat HCO_3^- levels did not exceed 70 mmol/L (24-28).

In humans, the production of pancreatic juice is strongly dependent on postprandial release of the hormone secretin, whereas production is low during fasting. Secretin, through activation of Gα,s-coupled receptors, triggers cyclic AMP (cAMP) production and a consequent protein kinase-mediated phosphorylation and activation of CFTR. In humans, CFTR-mediated, cAMP-dependent ductal fluid secretion is strongly potentiated by parasympathetic, vagal stimulation. Most plausibly, the activation of muscarinic (Gα,q) receptors by acetylcholine triggers K^+ efflux through Ca^{2+}-dependent K^+ channels, which sustains the negative electrical potential across the luminal plasma

membrane driving Cl^- and HCO_3^- efflux through CFTR. In addition to cholinergic neurons, the parasympathetic system comprises neurons that produce vasoactive intestinal polypeptide (VIP), which, like secretin, activates Gα,s-coupled receptors.

2.2. Molecular mechanisms of CFTR-dependent bicarbonate transport

HCO_3^- secretion involves the coordinated activity of ion transporters located at the basolateral and luminal membrane of pancreatic ductal cells (PDCs) (Figure 1). According to current models, which are discussed in detail elsewhere, uptake of HCO_3^- from the interstitial space is principally mediated by a Na^+/HCO_3^- cotransporter (NBCe1, *SLC4A4*) located in the basolateral plasma membrane (13, 29). This transport is driven by the steep electrochemical Na^+ gradient across the plasma membrane (generated by Na^+,K^+-ATPase activity), and allows for intracellular accumulation of HCO_3^- up to levels well above electrochemical equilibrium. In addition, HCO_3^- may be produced intracellularly from carbonic anhydrase-catalyzed hydration of CO_2, which enters the cells through diffusion (Figure 1). While the protons produced in this reaction are extruded across the basolateral plasma membrane via a Na^+/H^+ exchange mechanism, HCO_3^- is secreted across the apical membrane into the ductal lumen via the cooperate activity of the CFTR channel and the Cl^-/HCO_3^- exchanger(s) SLC26A6 and, more tentatively, SLC26A3 (13, 30-32). According to this model, which may also be relevant for other HCO_3^--secreting epithelia such as the intestinal and biliary, CFTR serves to facilitate Cl^-/HCO_3^- exchanger-mediated HCO_3^- secretion by extruding the Cl^- ions absorbed through the exchanger(s) (22, 30, 33, 34). As for CFTR, the activity of Cl^-/HCO_3^- exchangers is stimulated by cAMP and Ca^{2+} agonists, which implies that their activity is also controlled through neuro-endocrine stimulation of Gα,s- and Gα,q-coupled receptors (35). CFTR may not only couple functionally to SLC26A6, but also physically, through binding of its regulatory (R) domain to the sulfate transporter anti-sigma (STAS) domain of SLC26A6 (36, 37). This interaction was shown to promote Cl^-/HCO_3^- exchange, even in the absence of CFTR activity (22, 36).

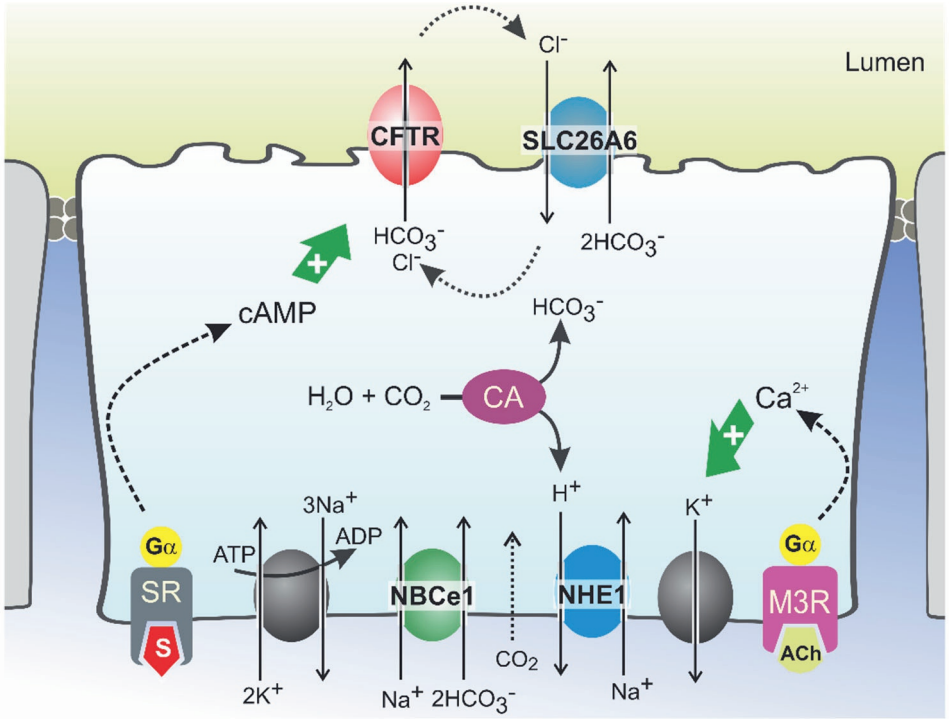


Figure 1. Model of HCO_3^- secretion in pancreatic ducts. HCO_3^- secretion is controlled by neuroendocrine input, chiefly by secretin (S) and acetylcholine (ACh), which increase cellular cAMP and Ca^{2+} levels, respectively, via stimulation of G-protein coupled receptors. This triggers Na^+-coupled HCO_3^- uptake across the basolateral plasma membrane through NBCe1 and luminal HCO_3^- efflux through CFTR and SLC26A6. HCO_3^- may also be accumulated intracellularly via the co-ordinate activity of carbonic anhydrases (CA) and a proton extrusion mechanism, NHE1. Opening of K^+ channels sustains the negative membrane potential which drives (CFTR-mediated) anion efflux. Cl^- ions absorbed via SLC26A6 may be recycled to the ductal lumen via CFTR. Ultimately, ductal ion and fluid secretion is driven by ion- and electrical gradients that are maintained by the Na^+,K^+-ATP-ase, located in the basolateral membrane. SR: secretin receptor, M3R: muscarinic acetylcholine receptor 3.

Although there is ample evidence for the role of SLC26-type Cl^-/HCO_3^- exchangers in ductal HCO_3^- secretion, mathematical modeling of the ion motive forces in ductal epithelium predicted that Cl^-/HCO_3^- exchange mechanisms only significantly contribute to cellular HCO_3^- extrusion up to luminal HCO_3^- levels of circa 70 mmol/L (29, 38). Consequently, SLC26-type HCO_3^- transport mechanisms may serve to secrete HCO_3^- in the proximal segments of the ductal tree, but to reach the final, high HCO_3^- levels observed in human pancreatic juice, an alternative transport mechanism must prevail in the distal part. In view of these considerations, it seems plausible that CFTR has a direct role in ductal HCO_3^- secretion. The CFTR channel itself was shown to directly mediate cellular HCO_3^- transport in studies on both cell models and epithelial tissues,

including pancreatic ducts (39-42). It has however also been noted that the permeability of the CFTR channel pore for HCO_3^- is 4-5 times lower than for Cl^- (39, 40, 43, 44). This implies that CFTR can only selectively secrete HCO_3^- when very low intracellular Cl^- and comparatively high HCO_3^- concentrations are attained. This situation may occur in the distal part of the ductal tree where, upon hormonal stimulation of pancreatic secretion, Cl^- levels in PDCs decrease to values as low as 5 mmol/L (29). In these settings, assuming that, concurrently, intracellular HCO_3^- is actively accumulated through NBCe1-mediated uptake, it is conceivable that CFTR may function primarily, if not exclusively, as a HCO_3^- transporter.

To account for the high HCO_3^- and low Cl^- levels found in pancreatic juice, it has further been proposed that the Cl^- over HCO_3^- conductance ratio of CFTR is not static, but can be lowered to allow selective HCO_3^- secretion (45-48). This dynamic regulation of CFTR anion conductance is thought to be mediated by members of the WNK (with no lysine) type of protein kinases (13, 18, 49, 50). WNK kinases (the family consists of 4 members) regulate an array of ion transporters through activation of downstream protein kinases OSR1 (oxidative stress-responsive kinase 1) and the highly homologous SPAK (STE20/SPS1-related proline/alanine-rich kinase) (51). The WNK kinases are thought to function as osmotic stress sensors and are activated by a lowering of intracellular Cl^- levels. Notably, WNK1 and WNK4 control renal electrolyte transport and mutations in either cause hypertension as a result of an unchecked tubular Na^+ re-absorption. In most cases, WNK kinases regulate the activity of ion transport mechanisms by controlling their localization in the plasma membrane.

At low extracellular Cl^- concentrations, WNK1-mediated OSR1 and SPAK activation was shown to markedly increase the HCO_3^- permeability of CFTR in CFTR transfected HEK293 cells and in guinea pig PDCs (52). Interestingly, WNK1 activation depended on CFTR function as the change in HCO_3^- permeability was not observed in cells expressing non-functional, mutant (F508del) CFTR, suggesting CFTR is required for lowering of the intracellular Cl^- concentration. More recently it was proposed that WNK1 increases the HCO_3^- permeability of CFTR by a direct interaction that does not require SPAK activation (Figure 2) (53). WNK kinases are also thought to reduce the surface expression of Cl^-/HCO_3^- exchangers of the SLC26A family (54). At high luminal HCO_3^- levels such as encountered in the distal ductal tree, this silencing of Cl^-/HCO_3^- exchange activity may prevent re-uptake of HCO_3^-, and, consequently, promote its net secretion. Paradoxically, both WNK1 and WNK4 were also shown to reduce CFTR protein levels at the cell surface and suppress CFTR-mediated anion efflux (55). Moreover, WNK kinases may also reduce NBCe1 activity, suggesting that their activation opposes CFTR-mediated HCO_3^- secretion (56). However, the inhibition imposed by the WNK/SPAK pathway on ductal fluid and HCO_3^- secretion is counteracted by the action of the scaffolding protein inositol-1,4,5-trisphosphate (IP_3) receptor-binding protein released

with IP_3 (IRBIT) (56). Through its PDZ domain, IRBIT co-localizes at the apical and basolateral plasma membrane with CFTR and NBCe1, respectively, and prevents WNK1/SPAK-mediated endocytosis by recruiting a protein phosphatase (PP1) that counters SPAK-mediated phosphorylation of CFTR and NBCe1 (56-58). In addition to its effect on surface expression, IRBIT may also increase CFTR open-channel probability (59). Recruitment of IRBIT to these molecular complexes appears to be stimulated through cAMP and Ca^{2+} signaling, and IRBIT is required for the synergism observed between these signaling pathways (60, 61).

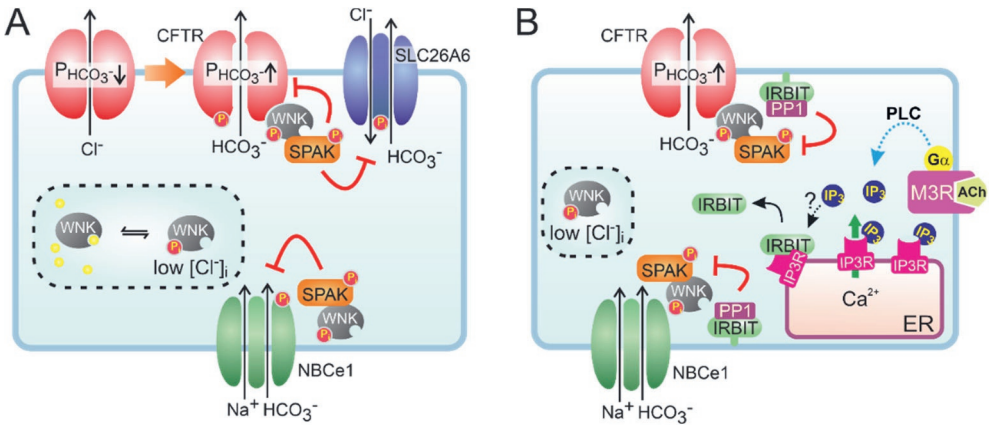


Figure 2. Hypothetical model of the role of WNK and IRBIT in ductal HCO_3^- secretion. (A) Activation of CFTR decreases intracellular Cl^-, which relieves the inhibition imposed by Cl^- on WNK and stimulates auto-phosphorylation of the protein kinase. Activated WNK binds to CFTR and increases the HCO_3^- permeability (P) of the channel pore, independent of its protein kinase activity. However, WNK also recruits SPAK, which leads to a phosphorylation-mediated decrease in the cell surface expression of HCO_3^- transporters, including CFTR. (B) IRBIT is bound to IP_3 receptors (IP3R) on cellular Ca^{2+} stores, e.g. the endoplasmic reticulum (ER). Conceivably, IRBIT is displaced from IP3R upon a phospholipase C (PLC)-mediated increase in IP_3 production, after which it translocates to the plasma membrane. Through its PDZ domain, IRBIT anchors to the actin cytoskeleton (not shown) and recruits a protein phosphatase (PP1) to the WNK/SPAK complex. PP1 activity counteracts SPAK-dependent phosphorylation of HCO_3^- transporters, thus stabilizing their expression at the cell surface. IRBIT does not affect the WNK-dependent, but SPAK-independent, increase in CFTR HCO_3^- permeability.

3. Bicarbonate transport in subjects with *CFTR* mutations

The past decade saw great advances in understanding the genetics of pancreatitis, including the role of *CFTR* mutations. Genes of which mutation confers an increased risk of developing (chronic) pancreatitis are grouped in 3 categories: (1) the trypsin-dependent pathway including *PRSS1*, *PRSS2*, *SPINK1*, *CTRC* and *CTRB1* (62-64); (2) the misfolding-dependent pathway including *CPA1* (65), *CEL* (66), and (3) the ductal pathway which, aside *CFTR*, includes *CLDN2* and *CASR* (5). *CFTR* mutations are

associated with development and earlier age of onset of RAP (67-69) and CP (70-73). In a large pediatric cohort, 34% of RAP and 23% of CP patients carried *CFTR* mutations (74). In a group of RAP patients with a mean age of 23 years, 15% carried *CFTR* mutations (69), and in a cohort of idiopathic CP patients this number was nearly 20% (72). Up to 7% of all CP patients carry *CFTR* mutations, and depending on the type of *CFTR* mutation, the risk for developing CP may increase 1.5 to 16 fold (75). This risk increases 3 to 21 fold when *CFTR* mutations are combined with a mutation in *SPINK1*, which encodes the pancreatic secretory trypsin inhibitor (75). The latter observation underlines the contention that pancreatitis is a multifactorial, complex disorder that develops through an interaction of multiple genetic and/or environmental factors. Consequently, the *CFTR* alleles that confer an increased risk of developing pancreatitis at the population level, only have a small effect on the individual risk (76).

Typical environmental factors that, in conjunction with genetic factors, modify the risk of pancreatitis are alcohol consumption and inhalation of tobacco smoke. As many as 40 to 70% of CP patients drink excessive amounts of alcohol (77-82). Similarly, smoking is an independent risk factor for onset and recurrence of AP (83) and 60% of CP patients regularly consume tobacco (75). *CFTR* mutations are more prevalent in alcohol and smoking related CP patients than in the general population, suggesting that partial loss of CFTR function aggravates the effect of such environmental factors (75). Rosendahl *et al.* elegantly depict the range of genetic and environmental interactions in different *CFTR* associated diseases (63). In CF and in hereditary forms of pancreatitis due to mutations in genes other than *CFTR* (like *PRSS1*), genetics are dominant and environmental factors have little influence on disease onset or severity. Conversely, for *CFTR* mutations that do not cause typical CF, the interplay with environmental risk factors becomes increasingly important.

To date, more than 2000 mutations in the *CFTR* gene have been described. These cover a wide spectrum, from apparently functionally silent mutations on one end, to the severe, CF causing mutations on the opposite end of the scale. Information on the pathophysiological and clinical consequences of these variants, has increased markedly over the last decade (1). The diagnosis CF requires two *CFTR* mutations on different alleles which severely impair CFTR channel function. Diagnosis of subjects who carry *CFTR* gene variants associated with residual protein function is often considerably less straightforward. The terms CF screen-positive, inconclusive diagnosis (CFSPID, European term) and CFTR-related metabolic syndrome (CRMS, North American term) both describe patients with elevated immune-reactive trypsinogen who carry no or only one apparently CF causing allele, which do not fulfil the diagnostic criteria for CF (84, 85). So-called CFTR-related disorders (CFTR-RD) are single organ diseases with evidence of CFTR dysfunction in the absence of CF diagnosis (1). CFTR-RD is commonly

associated with the presence of at least one *CFTR* allele with undefined clinical consequences.

3.1 Impaired pancreatic bicarbonate transport in CF patients

Impaired pancreatic HCO_3^- secretion in CF patients was recognized far before the discovery of the disease-causing gene (86). Follow-up studies indicated that both pancreatic HCO_3^- and Cl^- secretion were strongly reduced in patients and contribute to the CF-typical fluid secretory defect (87, 88).

As indicated previously, pancreatic dysfunction in CF is variable and correlates with genotype. In approximately 95% of the CF patients carrying severe *CFTR* mutations, i.e. resulting in a (truncated) immature protein that is not inserted into the plasma membrane (classes I, II), or in a channel with severely impaired gating (class III), the ion and fluid secretory function of the pancreatic duct is strongly impaired. This leads to atrophy of the ductal and, ultimately, also the acinar structures, culminating in extensive fibrosis and exocrine pancreatic insufficiency. In contrast, CF patients carrying "mild" class IV or V mutations are almost exclusively pancreatic sufficient (89). Generally, pancreatic sufficient CF patients also have a milder respiratory phenotype, lower mean sweat Cl^- concentrations and higher life expectancy than pancreatic insufficient CF patients (90). The latter group of patients displays overt fibrosis of the exocrine pancreas and a virtually complete loss of HCO_3^- and enzyme secretion. In the pancreatic sufficient CF group enzyme production is apparently adequate, but HCO_3^- secretion may nevertheless be significantly diminished (91, 92). Pancreatic sufficient CF patients are at an increased risk of developing pancreatitis, with a median age of onset at 18 years. Pancreatitis in this cohort is often precipitated by fatty meals and alcohol ingestion (93).

3.2 Bicarbonate and viscid mucus

It is well established that CFTR-dependent HCO_3^- secretion is essential for solubilization of mucins, i.e. the polymeric glycoproteins that form the main constituent of mucus, in most CF-relevant epithelia (94, 95). In the respiratory and the intestinal tract, loss of CFTR-mediated HCO_3^- secretion causes mucins to remain densely packed and attached to the epithelial surface. This leads to the formation of an abnormally viscid and strongly adherent mucus layer (96, 97). In the airways, a reduced HCO_3^- secretion and the concomitant accumulation of viscid mucus is also thought to affect pH regulation at the luminal surface, thought to be crucial for the defense against microbial colonization (98, 99). Furthermore, accumulation of hyper-viscid mucus impedes mucociliary clearance (100). So far, the importance of HCO_3^- in preventing mucus plugging and in anti-microbial defense in human pancreatic ducts has not been assessed, mainly because of the current lack of representative *in vitro* models and bona fide *in vivo* assays. However, ultrastructural and histochemical studies on autopsy pancreatic

tissue and in newborn CF pigs and ferrets demonstrated that early acinar plugs consist of zymogen material, not mucus, but that subsequent mucous metaplasia occurs as the obstruction and exocrine atrophy progress (101, 102). Therefore, acinus plugging in CF is primarily due to a defect in ductal fluid secretion, not to accumulation of viscous mucus.

3.3 Bicarbonate transport in subjects with non-CF-causing CFTR mutations

CFTR-RD includes diseases of the pancreas (i.e. acute recurrent or chronic pancreatitis), the male reproductive tract (congenital bilateral absence of the vas deferens) and the upper respiratory tract (i.e. chronic sinusitis) (103, 104). It has been suggested that, for proper function, these tissues are particularly dependent on CFTR-mediated HCO_3^- transport, and that the non-CF-causing *CFTR* mutations associated with these disease syndromes primarily affect the capacity of CFTR to mediate HCO_3^- transport (105, 106). Whether carrying a *CFTR* mutation on only one allele can give rise to these phenotypes is still unresolved (105).

In line with the concept that CFTR permeability can be dynamically regulated (Figure 2), it has been proposed that a selected group of *CFTR* mutations specifically reduce the permeability of CFTR to HCO_3^-. Nine such mutations were identified (*CFTR* R74Q, R75Q, R117H, R170H, L967S, L997F, D1152H, S1235R, and D1270N) in the North American Pancreatitis Study 2 cohort (105). Patients were screened for *CFTR* mutations typically not associated with severe CF. These mutations were not only associated with pancreatitis, but were additionally associated with rhinosinusitis and male infertility. They were termed bicarbonate-defective CFTR (CFTR-BD) because they showed normal Cl^- but decreased HCO_3^- permeability in response to WNK1-SPAK activation (105). Two of these mutations, R74Q and R75Q, showed a reduced association with WNK1 (53). However, more recent studies could not confirm that the R75Q mutation is a risk factor for CP; neither in the presence or absence of a concurrent *SPINK1* mutation (107).

4. Acquired CFTR dysfunction in pancreatitis

Pancreatitis may not only be linked to *CFTR* mutations, but may also result from environmental factors that reduce (wild type) CFTR function.

4.1 Alcohol

Early studies showed that alcohol precipitates pain attacks in pancreatic sufficient CF patients, as well as in patients suffering from hereditary pancreatitis and alcoholic CP (108). Low concentrations of ethanol (0.3-30 mmol/L) acutely increased secretin-stimulated fluid secretion of guinea pig PDCs (109), while high concentrations (100mmol/L) decreased ductal fluid and HCO_3^- secretion by reducing CFTR expression and function (17, 109). Increased sweat Cl^- levels suggested that CFTR function was

impaired in patients with excessive alcohol consumption (17). CFTR protein expression was decreased in pancreatic tissues of AP and CP patients obtained from autopsies and surgical resections (17). Ethanol or its metabolites (palmitoleic acid and palmitoleic acid ethyl ester) were also shown to decrease cellular cAMP and ATP levels in ductal cells, possibly by impairing oxidative phosphorylation (17, 110, 111). Accordingly, inhibition of the carboxylester lipase, a key enzyme in the non-oxidative ethanol metabolic pathway decreased the severity of alcoholic AP (112).

4.2 Bile acids

Biliary pancreatitis is the leading cause of AP in both children and adults (113). A proposed mechanism is the reflux of bile into the pancreatic duct, caused by gallstones or sludge within the distal common bile duct. Bile acid exposure causes pancreatic acinar cell injury through a sustained rise in cytosolic $Ca^{2+}$and activation of the Ca^{2+}-activated phosphatase calcineurin (114). Bile acids also exert a dose-dependent effect on PDC functions. A low concentration of chenodeoxycholate (CDC; 0.1 mmol/L) was shown to stimulate apical Cl^-/HCO_3^- exchange activity in guinea pig ducts (115) and CFPAC-1 cells only after *CFTR* expression (116). However, CDC administration did not activate the CFTR Cl^- channel (115, 116). In contrast, a high CDC concentration (1 mmol/L) inhibited HCO_3^- secretion in isolated guinea pig pancreatic ducts by causing severe mitochondrial damage (115, 117).

4.3 Smoking

Smoking is associated with multiple systemic disorders and leads to acquired CFTR dysfunction in the airways, sweat glands and intestine (118). The effects of smokers' plasma on bronchial epithelial cells suggested the involvement of a circulating component of smoke (118). Acrolein, nicotine, cadmium and manganese have been implicated in CFTR inhibition (118-121). While some report a transient activation of CFTR in airway epithelia (122), others show rapid internalization of CFTR from the plasma membrane (123-125). Importantly, the CFTR potentiator ivacaftor (VX-770) reversed the acute CFTR inhibition caused by cigarette smoke extract exposure in human bronchial epithelial cells (126).

Smoking is also an independent risk factor for the development of chronic pancreatitis (79). Past and current smokers had lower secretin-stimulated peak HCO_3^- concentrations in pancreatic fluid, indicating impaired CFTR mediated pancreatic ductal secretion (127). Cigarette smoke extract seemed to inhibit CFTR activity and HCO_3^- secretion in guinea pig pancreatic ducts (119). Smoking elevated sweat Cl^- concentrations in CP patients and decreased CFTR protein expression at the cell surface (128). Incubation with cigarette smoke extract decreased CFTR expression in CAPAN1 cells and HCO_3^- secretion in guinea pig pancreatic ducts (128). Whether this decrease in HCO_3^- secretion can be reversed by ivacaftor is not known but worth investigation.

4.4 Susceptibility to pancreatitis inducers

Since not all patients exposed to established environmental risk factors develop pancreatitis (e.g. the majority of heavy alcohol users do not develop pancreatitis), it is probable that additional (genetic) factors play a role. CFTR may be one of those genetic modifiers. Children diagnosed with pancreatitis who were exposed to smoke and had *CFTR* mutations were admitted to the hospital more often than children without *CFTR* mutations (129). In patients with wild type *CFTR*, a reserve CFTR activity may compensate for the deleterious effects of alcohol, but in patients carrying CFTR mutations, alcohol consumption may compromise CFTR function sufficiently to trigger symptomatic disease. Non-CF causing *CFTR* mutations are also included in experimental pancreatitis models and underline the essential role of CFTR function in PDC (patho-)physiology. In transgenic mice with impaired Cl^- transport but significant residual CFTR function ($CFTR^{tm1HGU}$), the severity of cerulean-induced pancreatitis was increased, mostly via impairment of PDC function and a shift towards a pro-inflammatory phenotype (130).

5. CFTR modulators

Whereas symptomatic therapy of CF is only able to ameliorate the pathological consequences of CF, CF modulator therapy directly targets the roots of CF, namely CFTR dysfunction (131-133). Modulators are pharmacological compounds that can be classified on the basis of their different mode of action. So-called potentiators, e.g. ivacaftor (VX-770), restore CFTR channel gating; correctors, e.g. lumacaftor (VX-809), tezacaftor (VX-661), and elexacaftor (VX-445), improve CFTR folding and trafficking to the cell surface and some, like elexacaftor, may also act as co-potentiators (133); amplifiers, e.g. PTI-CH, co-translationally enhance CFTR biosynthesis by stabilizing *CFTR* mRNA (134); and read-through agents for *CFTR* nonsense mutations, e.g. aminoglycosides (135) and analogs (ELX-02) (136, 137), or the recently developed compound SRI-37240 suppress premature termination codons (138). The spectrum of mutations for which each modulator is clinically approved, was reviewed recently (139).

5.1 Modulator effects in the pancreas

So far only a few studies have addressed the effectiveness of CFTR modulators in animal models of pancreatitis. In a murine AP model induced by cerulean, pretreatment with tezacaftor and ivacaftor reduced the extent of tissue damage but did not affect other parameters, while *in vitro* administration increased fluid secretion in pancreatic ducts of AP animals (140). Furthermore, in a murine autoimmune pancreatitis model, the CFTR corrector C18 rescued CFTR expression and localization in the pancreas (141). Although these model studies provide valuable insight into the course of CFTR function and expression during pancreatitis, species differences should be considered, not only

in epithelial physiology but also in drug sensitivity. For example, ivacaftor and multiple other potentiators have been shown to act on human but not murine F508del CFTR (142).

Recent studies of ivacaftor with or without lumacaftor contradicted the assertion that exocrine pancreatic insufficiency is irreversible in CF children (143-145). However, while pancreatic function improved, as evidenced by increased fecal elastase-1 concentration and decreased immunoreactive trypsinogen levels, some studies reported an increase in pancreatitis episodes, resembling the phenotype of pancreatic sufficient CF patients (143, 144). However in pancreatic sufficient CF adults, CFTR modulator therapy reduced the risk and frequency of RAP (146, 147). One study included 1800 CF patients who received CFTR modulators and showed a 67% and 62% reduction in AP hospitalizations in pancreatic sufficient and pancreatic insufficient CF patients, respectively (148). These results suggest that early treatment with CFTR modulators might prevent pancreatic damage, and that CFTR modulators can ameliorate pancreatic complications of CFTR dysfunction. Interestingly, a recent case report documents a patient with RAP and a R117H/7T/F508del *CFTR* genotype, without respiratory symptoms but with elevated sweat Cl^-, who was effectively treated with ivacaftor (149). This suggests a role for *CFTR* genotype testing in pancreatitis patients to identify those who could benefit from CFTR modulator therapy.

5.2 Modulator effects on HCO_3^- transport

Multiple *in vitro* and a few *in vivo* studies report various effects of CFTR modulators on HCO_3^- transport. For example, telemetric measurements of luminal pH in the duodenum of CF patients carrying the G551D gating mutation showed a profound increase in duodenal pH upon ivacaftor treatment, but in this study the origin of the HCO_3^- (intestinal, biliary or pancreatic duct) is unclear (150). In another *in vivo* study, ivacaftor/lumacaftor treatment of CF patients increased renal pendrin (*SLC26A4*)-mediated HCO_3^- excretion through correction of F508del CFTR activity in the β-intercalated cells of the collecting duct (37). Interestingly, surface liquid alkalization in primary human airway epithelial cell cultures triggered by a triple combination of ivacaftor, elexacaftor and tezacaftor (marketed as Trikafta) was likewise pendrin-mediated, but in this cell type pendrin expression was dependent on pre-treatment with the pro-inflammatory cytokines TNFα and IL-17 (151). In Fischer rat thyroid cells, a common model to study CFTR function, the CFTR corrector lumacaftor was shown to increase the HCO_3^- permeability of F508del-CFTR, and surprisingly to a greater extent than its Cl^- permeability (152). Similarly, ivacaftor was reported to repair the specific HCO_3^- conductance defect of the D1152H CFTR-BD mutation in monolayers of human nasal epithelial cells (153).

5.3 Modulator studies in pancreatic ductal organoids

Organoids generated from stem cells in intestinal biopsies and cultured in an extracellular matrix (3D) or as monolayers (2D) on filters or on microfluidic chips have been used as novel tools to study epithelial (patho-)physiology and to predict clinical effects of CFTR modulators in individual patients (personalized medicine) (154-156). Recently, culturing methods have been adapted to allow a similar long-term expansion of mouse and human pancreatic ductal organoids (PDOs) (157-159). Pancreatic organoids can be generated from adult stem cells in pancreatic juice collected during endoscopic ultrasound (160), from microdissected animal- or patient-derived pancreatic ducts (159, 161) or, possibly, even from microbiopsies collected during ERCP (162). PDOs can be cryopreserved, clonally expanded, and genetically manipulated, and retain the characteristics of the tissue of origin, including gene expression and function of ion channels and transporters (157, 161). They can be grown 2D as polarized monolayers on filters or chips, offering highly suitable models to study Cl^- and HCO_3^- transport pathways and their regulation (159, 161) (Figure 3). However, it will be a challenge to reproduce the unique ability of PDCs to accumulate HCO_3^- up to a 140 mmol/L concentration at the luminal bath.

Human PDOs may also facilitate drug development and drug screening for pancreatic disorders like CF or genetic and acquired forms of AP or CP. One restriction is the poor availability of human biopsies or tissue explants from the pancreatic duct of CF, CFSPID or CFTR-BD patients. In this case creating a human model to study the effect of CFTR mutations and modulators on PDC properties and function would require gene editing of CFTR in non-CF PDOs. Additionally, it would be of great interest to study the ability of CFTR modulators to restore CFTR function in PDCs exposed to alcohol, tobacco smoke or bile salts. In particular the therapeutic potential of VX-770 in a human PDO model deserves further investigation, considering the strong potentiating effect of this drug on wild type CFTR channels (163).

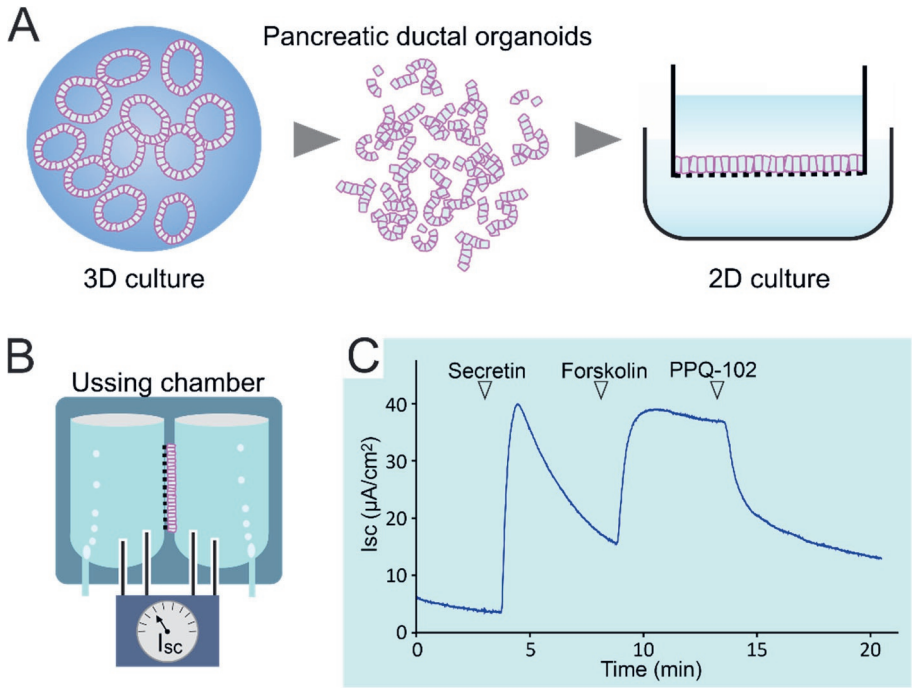


Figure 3. (A) Pancreatic ducts were isolated from porcine pancreas by enzyme treatment and suspended in an extracellular matrix. From these ductal structures, spherical organoids are formed within a few days. Cultures are renewed and expanded by mechanical disruption and reseeding of the resulting cell clusters. These can also be used to initiate epithelial monolayers (2D cultures). (B) When cultured on a permeable substrate, monolayers can be used to asses epithelial anion transport in an Ussing chamber, analogous to ICM. (C) Current (Isc) response of a pancreatic organoid-derived monolayer upon stimulation by secretin and the cAMP agonist forskolin, and the effect of the CFTR blocker PPQ-102.

6. Concluding remarks

Whereas complete loss of CFTR function leads to the CF-typical fibrosis of the exocrine pancreas and pancreatic insufficiency, it has become clear in recent years that milder forms of CFTR dysfunction, whether congenital or acquired, are involved in the pathophysiology of pancreatitis. Congruently, recent studies suggest that CFTR modulators originally developed for CF therapy, may also be of potential benefit in this context. However, not all patients suffering from pancreatitis carry CFTR mutations and not all CFTR mutations may be amenable to correction. Therefore, it will be important to identify those CFTR variants that are potentially responsive to drug therapy. PDOs provide a promising model to achieve this objective, as they may be used to select modulators on a personalized basis. Finally, clinical testing of CFTR modulators is indicated to further clarify the role of CFTR dysfunction in the development of AP and CP, and to validate the therapeutic potential of CFTR modulators.

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Chapter 3

Evaluation of the response to elexacaftor-tezacaftor-ivacaftor of the rare CFTR variants L383S, I507del, L1065P and R1066H in intestinal organoid-derived epithelial monolayers

Jessica Conti[1$], Dora Angyal[2$], Karina Kleinfelder[1], Roberta Valeria Latorre[1], Martina Calicchia[1], Alessia Farinazzo[1], Luca Rodella[3], Francesco Tomba[3], Arianna Massella[3], Luca Frulloni[4], Giovanni Taccetti[5], Vito Terlizzi[5], Anny Leung[2], Tessa A. Groeneweg[2], Marcel J.C. Bijvelds[2$], Paola Melotti[6], Claudio Sorio[1$*]
[$]Equal contribution.

[1]Department of Medicine, University of Verona, Division of General Pathology, Cystic Fibrosis Laboratory "D. Lissandrini", Verona, Italy
[2]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
[3]Endoscopy Surgery Unit, Azienda Ospedaliera Universitaria Integrata Verona, 37126 Verona, Italy.
[4]Gastroenterology Unit, Department of Medicine, Borgo Roma Hospital, Piazzale L.A. Scuro 10, 37134 Verona, Italy
[5]Meyer Children's Hospital IRCCS, Cystic Fibrosis Regional Reference Center, Department of Paediatric Medicine, Florence, Italy (G.T., V.T.);
[6]Cystic Fibrosis Centre, Azienda Ospedaliera Universitaria Integrata Verona, Verona, Italy

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Part I

Abstract

Introduction: Cystic fibrosis (CF) is caused by mutation of the *CFTR* gene, encoding an epithelial anion channel. Here, we evaluated the effect of the modulator combination elexacaftor-tezacaftor-ivacaftor (ETI) on the function of four rare, poorly characterized CFTR variants: L383S, I507del, L1065P and R1066H.

Methods: Intestinal organoids were obtained from subjects carrying the CFTR variants L383S, I507del, L1065P or R1066H *in trans* of a minimal function allele (class I mutation). Organoids and epithelial monolayers were used to assess the effect of ETI on CFTR protein abundance and CFTR-mediated chloride, bicarbonate, and fluid transport.

Results: In L383S-CFTR expressing cells, normal levels of fully glycosylated CFTR protein (C-band) were detected. In contrast, in I507del, L1065P or R1066H organoids, only partially glycosylated CFTR (B-band) was detected. Chloride/bicarbonate transport was severely impaired in epithelial monolayers prepared from the latter three variants, while anion transport of the L383S variant was affected to a moderate extent. ETI, but not ivacaftor alone, significantly enhanced CFTR-mediated chloride and bicarbonate transport in L1065P and R1066H monolayers, and stimulated fluid transport. A corresponding increase in the abundance of C-band protein was observed in both variants. ETI also modestly improved L383S-CFTR function, with a marginal effect on I507del-CFTR.

Conclusions: The I507del, L1065P and R1066H variants display severely impaired function. ETI treatment markedly enhanced L1065P- and R1066H-CFTR function, whereas its effect on L383S-CFTR was less pronounced. Consequently, ETI may ameliorate disease symptoms in individuals carrying the L1065P or R1066H variant. More tentatively, it may also benefit those carrying the L383S variant.

Keywords: Rectal organoids; cystic fibrosis; theratyping; Ussing chamber; personalized medicine; CFTR variants, bicarbonate secretion.

1. Introduction

Cystic fibrosis (CF) is a potentially lethal disease that is caused by mutations of the CF transmembrane conductance regulator (CFTR) gene. *CFTR* encodes a phosphorylation-regulated chloride/bicarbonate channel that drives salt and water secretion across, principally (but not exclusively), epithelia in the respiratory and gastrointestinal tract. CF pathology is characterized by luminal accumulation of viscous mucus in the bronchi of the lungs, in the intestines, and the biliary and pancreatic ductal tree, promoting luminal obstruction, microbial colonization and inflammation (1). In particular the loss of CFTR-dependent bicarbonate secretion and the resulting luminal acidification is thought to precipitate the formation of an abnormally viscid mucus, by impeding the proper unfolding and release of mucins (2, 3).

Whereas up to quite recently management of CF relied principally on supportive care and symptomatic treatment, the development of CFTR modulators has dramatically altered the treatment of CF. Modulators are small-molecule compounds that, by directly binding to the CFTR protein, improve its channel function. Generally, two types of modulators are distinguished: *correctors* increase the amount of CFTR in the cell membrane by enhancing co-/posttranslational protein folding and trafficking, whereas *potentiators* improve the gating of the mutant channel, increasing the flow of anions through the channel pore. In clinical trials, a drug combination composed of two correctors (elexacaftor and tezacaftor) and one potentiator (ivacaftor), has demonstrated unprecedented improvements in disease parameters (4, 5). This triple combination (ETI) was initially approved in in the U.S. (brand name: Trikafta) and the E.U. (Kaftrio) for people with CF (pwCF) ≥6 years of age, carrying one or two copies of the most common disease causing allele, F508del-CFTR. It has been estimated that circa 90% of pwCF in these geographical regions carry this allele, and are eligible for ETI therapy (6). In addition, circa 3% of pwCF carry CFTR variants that confer a channel gating defect which can be restored by potentiator therapy alone (6).

For many other, mostly very rare, CFTR variants, the efficacy of modulator therapy is largely uncharted. Because the number of pwCF carrying a mutation of this category is inevitably highly limited, clinical trials are impractical, and up until recently, pwCF carrying such mutations did not readily have access to modulator therapy (7). However, based on studies in which the effect of modulators on CFTR function was evaluated in Fisher rat thyroid (FRT) cells transfected with CFTR, the U.S. Food and Drug Administration (FDA) has since notably extended the group of approved variants (8, 9).

An alternative approach to testing the response of rare CFTR variants to modulator treatment is the use of organoid-based assays (10-15). These assays are based on the culture of (typically) primary respiratory or intestinal epithelial stem cells, obtained from individual pwCF. In this approach, as opposed to the FRT model, the efficacy of

modulator treatment is evaluated in the appropriate genomic context, and, in general, the outcome of such assays seems to correspond well with clinical efficacy (4, 16, 17).

The aim of this study was to evaluate the effect of ivacaftor (IVA) and ETI on the rare CFTR variants L383S (c.1148T>C), I507del (c.1519-1521delATC) and L1065P (c.3194T>C); estimated allele frequencies within the CF population: <0.001%, <1% and 0.03%, respectively (www.cftr2.org, accessed March 2024). At present, these CF alleles are not eligible for modulator therapy. We generated intestinal rectal organoids from three pwCF that carried either one of these variants *in trans* of a minimal function allele harboring a class I mutation (a reading frame shift, exon deletion or nonsense mutation), unresponsive to modulators. Consequently, any potential beneficial effects of IVA or ETI observed in our assays must be attributed to the rescue of the other allele. In addition to these three poorly characterized variants, we included an individual carrying the R1066H (c.3197G>A) variant *in trans* of the class I mutation R1162X in our analysis. R1066H is also a rare CFTR variant (estimated allele frequency: 0.07%), but its functional defects, as well as its response to IVA and ETI, have been investigated previously in the FRT model as well as in intestinal organoids (14, 18, 19).

Presently, we assessed CFTR-dependent fluid transport in organoids, and prepared epithelial monolayers from these organoids, to assess the effect of IVA and ETI on CFTR-mediated anion transport, using a modification of the intestinal current measurement (ICM) protocol, typically used to assess CFTR function in intestinal biopsies (15, 20, 21). Like the aforementioned assays on FRT cells, presently exploited to characterize the response of rare CFTR variants to modulators, this technique assesses CFTR-mediated anion transport across an epithelial monolayer (18, 19). Importantly, because some CFTR variants were shown to have a selective defect in bicarbonate conductance, and because modulators may affect bicarbonate and chloride conductance of the channel pore differently, we adapted the ICM technique to allow separate measurement of CFTR-mediated chloride *vs*. bicarbonate transport (22, 23).

2. Materials and methods

2.1 Tissue collection

Subjects were recruited at the Cystic Fibrosis Centre, AOUI Verona and Meyer Children's Hospital, Verona. Study protocols were approved by the local ethical committee (CRCFC-CFTR028) and written informed consent was obtained from all participating subjects.

Rectal tissue was obtained using a biopsy forceps. After collection, biopsies were placed in RPMI-1640 medium (Gibco) supplemented with glutamax supplement (1:100; Gibco), HEPES (10 mmol/L), and a mixture of antibiotics (penicillin, streptomycin, gentamicin and ciprofloxacin).

2.2 Organoid culture

Rectal organoids were cultured as described in detail elsewhere (24). In brief, rectal biopsies were washed in phosphate buffer saline (PBS) and subsequently incubated in PBS supplemented with EDTA (10 mmol/L) for 20-90 min at 4 °C, to collect crypts. Isolated crypts were suspended in extracellular matrix (Matrigel, Corning) and cultured in a medium containing the growth factors Wnt3a, Noggin, R-Spondin 1 and EGF. The culture medium was supplemented with Y27623 and CHIR-99021 (10 µmol/L each; Merck) during the first two days after seeding. Additional antibiotics (gentamycin and vancomycin, both at 50 µg/mL; Sigma) were added to the culture medium during the first week after the seeding of crypts. Brightfield micrographs of organoid cultures were acquired using an EVOS FL cell imaging system (4x objective; Invitrogen).

2.3 Culture of epithelial monolayers

Epithelial monolayers were generated from organoids as previously described (15, 20). Briefly, after cryopreserved organoids were thawed, the different lines were expanded by sub-cultivation (1:5) over 3-4 cycles. Organoid-derived cells were seeded on filter inserts (250,000/cm^2; Transwell #3470, Costar). The same culture medium was used as for extracellular matrix-embedded organoids. Before seeding, filter inserts were treated with collagen type IV (10 µg/cm^2; Sigma) dissolved in PBS for 2h at 37°C. Formation of epithelial monolayers was monitored by measuring of the transepithelial electrical resistance (TEER) using an EVOM Epithelial Voltohmmeter (World Precision Instruments). Epithelial monolayers were cultured for >3 weeks, resulting in TEER values typically >200 Ω.cm^2.

2.4 Fluid transport in organoids

CFTR-mediated anion secretion stimulates osmotic fluid transport into the enclosed lumen of intestinal organoids, leading to organoid swelling (14). To evaluate fluid secretion, organoids were seeded in 96-well cell culture plates in droplets (3 µL) of

extracellular matrix (Matrigel). From 24h after seeding, organoids were cultured for 24h, in the presence of ELX (VX-445; 3 µmol/L; Medchem Express) and TEZ (VX-661; 3 µmol/L; Selleckchem) or vehicle (0.2% DMSO). Subsequently, cells were incubated with calcein AM (5 µmol/L, 30 min, Invitrogen), and CFTR-dependent organoid swelling was triggered by addition of forskolin (10 µmol/L) in the presence or absence of the CFTR potentiator IVA (VX-770; 0.3 µmol/L; Selleckchem). Live-cell imaging was performed using a Leica TCS SP5 confocal microscope (Ex=488 nm, 5x objective), equipped with a cell incubation system (37°C, 5% CO_2; Leica Microsystems). Micrographs were captured at 6 min intervals for 1h using LAS AF software (Leica Microsystems). The total area occupied by the fluorescently labeled organoids in each droplet/well (containing circa 30 organoids) was quantified on the ImageJ platform (https://imagej.net), using a software module that fully automates image analysis.

2.5 Chloride and bicarbonate transport across epithelial monolayers

Prior to current measurements, monolayers were incubated with ELX (3 µmol/L; Medchem Express) and TEZ (3 µmol/L; Selleckchem) or with vehicle (0.2% DMSO), for 24h. Experiments were designed to allow for a paired comparison, i.e. the monolayers used for assessing the response to CFTR modulators or vehicle were cultured and assayed in parallel. Subsequently, filter inserts were mounted in an Ussing chamber (P23023T/P2300, Physiologic Instruments). For assessing CFTR-mediated chloride transport, monolayers were bathed in (mmol/L) 128 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 Na-isethionate, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose. For assessing CFTR-mediated bicarbonate transport, monolayers were bathed in (mmol/L) 128 Na-isethionate, 4.7 K-isethionate, 1.3 $Ca(CH_3COO)_2$, 1.0 $Mg(CH_3COO)_2$, 20 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose. The only CFTR-permeating anion in this latter solution is bicarbonate. Bathing solutions were maintained at 37°C and gassed continuously with O_2 or 95% O_2/5% CO_2, respectively. When applicable, the CFTR modulators or DMSO were added to the bathing solutions. The potential difference across epithelial monolayers was set at 0 mV using a VVC-MC8 voltage clamp module (Physiologic Instruments), and short-circuit currents (Isc) were recorded using LabChart 8 software (AD Instruments). After the Isc had reached a steady state (ca. 5 min), baseline Isc values were recorded. Addition of the cAMP agonist forskolin (10 µmol/L) was used to activate CFTR channel activity, followed by IVA (0.3 µmol/L; Selleckchem).

2.6 Western blot analysis

Organoids, cultured for 6 days, were treated (24h) with ELX (3 µmol/L) and TEZ (3 µM), or with vehicle (DMSO). Cells were collected in cell recovery solution (Corning), and subsequently lysed in NaCl (150 mmol/L), Tris/HCl pH 7.6 (25 mmol/L), Triton X100 (1%), sodium deoxycholate (1%), sodium dodecyl sulfate (0.1%), supplemented with a

protease inhibitor cocktail (Roche). After the protein concentration was determined using a reagent kit (Bio-Rad), lysates (40 μg protein per CF line and 20 μg for the non-CF line) were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membrane. For detection of CFTR, a combination of 3 monoclonal antibodies (#450, #570, #596; 1:3000 each) was used, kindly provided by the Cystic Fibrosis Foundation, through the CFTR antibody distribution program (University of North Carolina**,** Chapel Hill; https://cftrantibodies.web.unc.edu/). Detection of E-cadherin (Cell Signaling #24E10) was used as a loading control. Primary antibodies were detected using fluorescently labeled secondary antibodies (goat-anti-mouse- or goat anti-rabbit IgG; Invitrogen #A21057 and #A32735, respectively). Fluorescence was detected and analyzed using an Odyssey imaging system and associated software (Image Studio Lite 5.2; Licor Biosciences).

2.7 Statistical analysis

Data are expressed as means ± S.D. Ordinary one-way ANOVA (IBM SPSS 28.0) was used to determine the statistical significance of differences between means. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1 Subject description

Of the four pwCF included in this study, three carried rare CFTR variants of which the function and response to modulator treatment has, to our knowledge, not been studied before (L383S), or only in a heterologous expression system, i.e. FRT cells (I507del, L1065P). Subjects 2 (I507del/2183AA>G) and 3 (L1065P/CFTRdele2) presented with sweat chloride levels >60 mmol/L, which are pathognomonic for CF, bronchiectasis, and pancreatic insufficiency, indicating that these variant combinations confer a comparatively severe disease phenotype (Table 1).

Table 1. Main clinical characteristics of CF subjects (all male).

Subject	Genotype	Age (years)	BMI	Lung coloni-zation	Bronchi-ectasis	Sweat chloride (mmol/L)	FEV1 (%)	Pancreatic function
1	[L383S] [2183AA>G]	49	31.6	MSSA	No	44	66	PS
2	[I507del] [2183AA>G]	33	22.5	Bc, Ax	Yes (before LTx)	109	89	PI
3	[L1065P] [CFTRdele 2]	17	22.6	MSSA	Yes (grade 1)	98	82	PI
4	[R1066H] [R1162X]	55	26.5	MRSA	Yes	112	77	PS

BMI, body mass index; Bc, *Burkholderia cenocepacia*; Ax, *Achromobacter xilosoxidans*; FEV1, forced expiratory volume in 1s relative to normal; MSSA, Methicillin-sensitive *Staphylococcus aureus*; MRSA: Methicillin-resistant *Staphilococcus aureus*; LTx, lung transplantation; PI, pancreatic insufficient; PS, pancreatic sufficient.

The L383S variant has not yet been described in the CFTR2 database. It is a highly uncommon variant (allelic frequency <0,001%) with largely unknown functional and clinical significance. The present individual presented with inconclusive sweat chloride levels (i.e. sweat chloride exceeded the normal range, but did not reach levels considered pathognomonic for CF) and pancreatic sufficiency. However, although no bronchiectasis was observed (at age 49), MSSA colonization of the airways was detected, and his FEV1 was lowered.

Although also rare, the functional consequences of the R1066H-CFTR variant, and its response to ETI, have been recently described (14, 18). Subject 4 (R1066H/R1162X) presented with CF-typical high sweat chloride levels, bronchiectasis, and MRSA colonization of the lungs, but also pancreatic sufficiency.

3.2 Organoid morphology

One day after seeding, the morphology of the organoids prepared from pwCF carrying the I507del-, L1065P-, or R1066H-CFTR variant was clearly distinct from the spheroid structures formed by cells expressing wild type (WT) CFTR, which, as a result of CFTR-dependent osmotic water transport, display a large fluid-filled lumen (Fig. 1A). Only the L383S-CFTR variant still formed proper spheroids with a lumen. In contrast, I507del-, L1065P- or R1066H-CFTR carrying organoids did not have a clearly discernable lumen and were irregular in shape, suggestive of a strongly impaired CFTR function. Closer inspection indicated that the area occupied by the lumen of L383S-CFTR expressing organoids, relative to their total area, is reduced when compared to organoids expressing WT-CFTR, i.e. the cells that line the lumen appear to occupy more of this total area (Fig. 1B). This observation points to a more moderate reduction in CFTR-dependent luminal fluid accumulation (14).

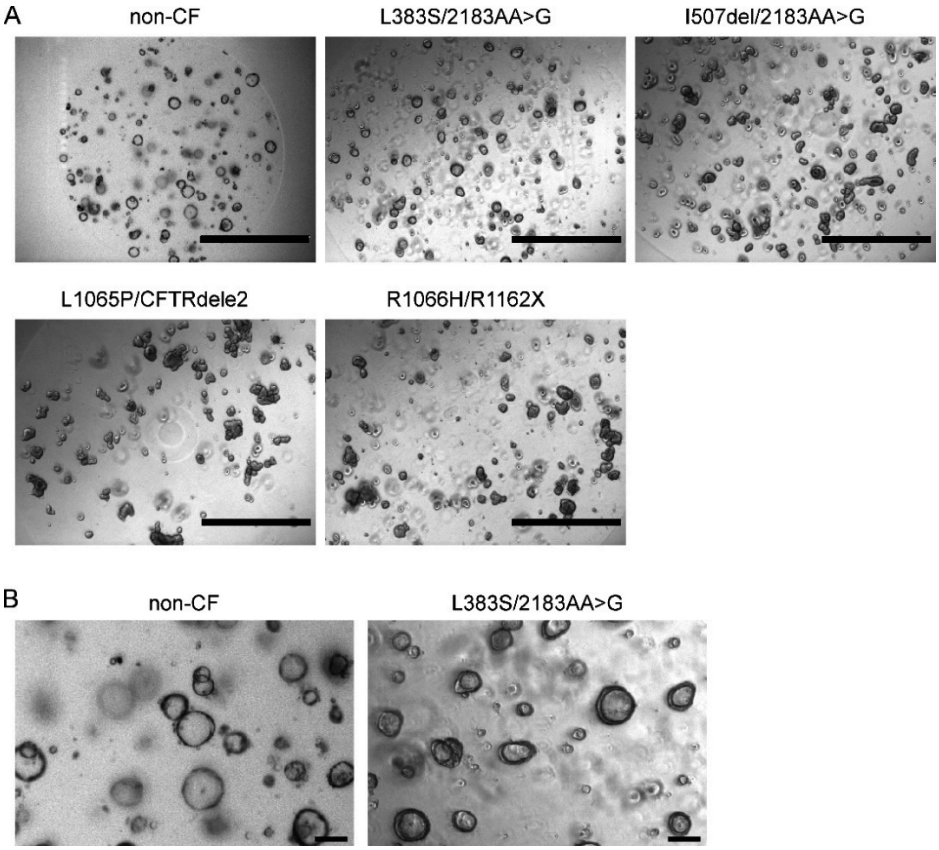


Figure 1. Morphology of CF and non-CF intestinal organoids. (A) Representative brightfield micrographs of organoids, one day after seeding. Organoids were obtained from subjects with *CFTR* genotype as indicated. Scale bar: 1 mm. (B) Detail of micrographs shown in panel A. Scale bar: 0.1 mm.

3.3 Effect of elexacaftor/tezacaftor treatment on mutant CFTR protein abundance

We assessed CFTR protein abundance in intestinal organoids by Western blot analysis. In all organoid lines investigated, the CFTR allele of interest was *in trans* of an allele carrying a class I variant, i.e. a coding sequence that results in the production of unstable and/or truncated mRNA that precludes protein synthesis. Further, because class I alleles are not amenable to correction of a protein trafficking defect, any effects of modulator treatment observed in our analysis must be attributed to an action on the product encoded by the other allele.

In organoids expressing WT-CFTR, a diffusive immune-reactive band was observed with a molecular mass of ca. 170 kDa, distinctive of the mature, fully glycosylated CFTR protein (C-band). A similar pattern was observed in organoids expressing the L383S-CFTR variant, suggesting that protein synthesis, and posttranslational processing in the endoplasmic reticulum and Golgi apparatus is not significantly impaired by this mutation (Fig. 2). In contrast, in organoids expressing CFTR variants I507del, L1065P or R1066H, no fully matured product could be detected. However, in all three, a faint immuno-reactive signal was detected with an apparent molecular mass of ca. 130 kDa, indicative of an incompletely glycosylated product (B-band), which is typically confined to an intracellular membrane compartment.

Next, we asked whether treatment with the correctors elexacaftor (ELX) and tezacaftor (TEZ) could improve the maturation of these variants. Indeed, in organoids expressing L1065P- or R1066H-CFTR, ELX/TEZ treatment (24h) markedly increased the level of fully matured CFTR protein (C-band). ELX/TEZ also tended to increase C-band protein levels in L383S-CFTR organoids. In contrast, ELX/TEZ treatment did not lead to a detectable increase in the level of C-band protein in I507del-CFTR expressing organoids.

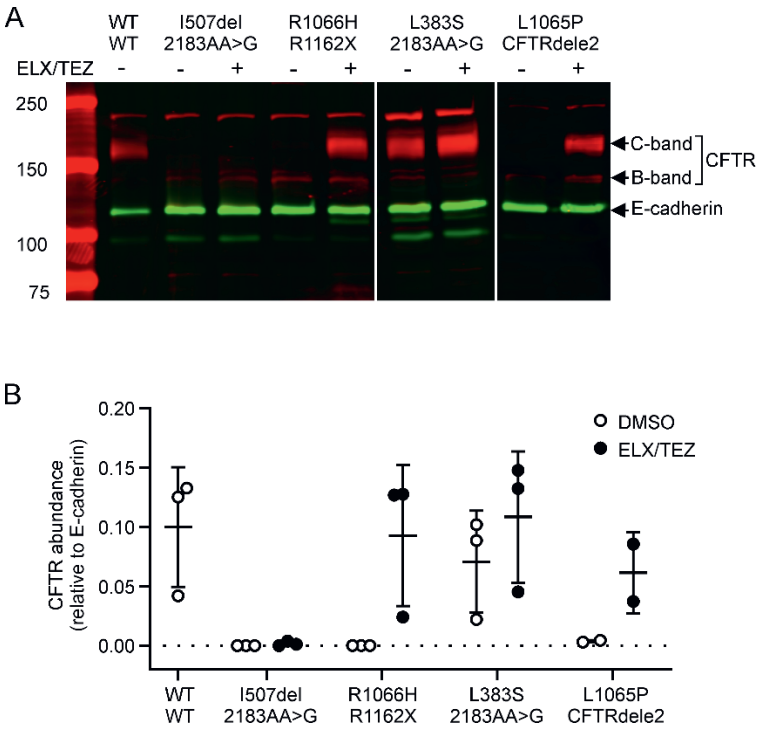


Figure 2. CFTR abundance in rectal CF organoids in the presence or absence of CFTR modulators. (A) Organoids from four pwCF were treated with elexacaftor and tezacaftor (ELX/TEZ) or vehicle (DMSO) for 24h, and CFTR protein was detected by Western blot analysis. For comparison, organoids expressing wild type CFTR (WT/WT) were loaded. Outer left lane shows markers with molecular weight as indicated (kDa). Detection of E-cadherin served as a loading control. B-band: partially glycosylated CFTR, C-band: fully glycosylated CFTR. (B) CFTR abundance in the presence or absence of ELX/TEX, relative to E-cadherin. Each data point represents one technical replicate.

3.4 Effect of elexacaftor/tezacaftor/ivacaftor treatment on CFTR-mediated chloride and bicarbonate transport

The effect of ETI on forskolin-dependent CFTR-mediated epithelial chloride and bicarbonate transport was studied in epithelial monolayers cultured from intestinal organoids, using a modification of the ICM protocol (15, 20). For comparison, we also assessed CFTR activity in intestinal epithelial monolayers derived from organoids expressing WT-CFTR or F508del-CFTR. In ETI-treated monolayers carrying the latter mutation, CFTR-mediated chloride secretion amounted to 29% of the response measured in monolayers expressing WT-CFTR (Table 2). This value is similar to the previously reported response of F508del-CFTR to ETI in the FRT model (22% of WT-CFTR), assessed under comparable conditions, i.e. in a medium containing chloride, but without bicarbonate (18). In bicarbonate medium, the response of ETI-treated F08del-CFTR monolayers was 45% of WT.

Epithelial monolayers expressing L383S-CFTR showed a high residual channel function, in accordance with the spheroid morphology of the organoids. The forskolin-dependent Isc was 76% and 80% of WT, in chloride and bicarbonate medium, respectively (Fig. 3, Table 2). Treatment with ETI modestly enhanced CFTR-mediated chloride secretion. IVA alone also tended to slightly increase Isc responses, although this apparent effect failed to reach statistical significance (P=0.09).

I507del and L1065P monolayers demonstrated a severely reduced Isc response to forskolin. ETI treatment markedly enhanced both CFTR-mediated bicarbonate and chloride transport in L1065P-CFTR monolayers. In contrast, IVA, in the absence of ELX and TEZ, did not have an effect on the forskolin-dependent Isc response. In I507del-CFTR monolayers, ETI treatment increased the Isc response in chloride medium from <1% to circa 2% of WT monolayers. No improvement of bicarbonate transport was observed. IVA, in the absence of ELX and TEZ, had no effect.

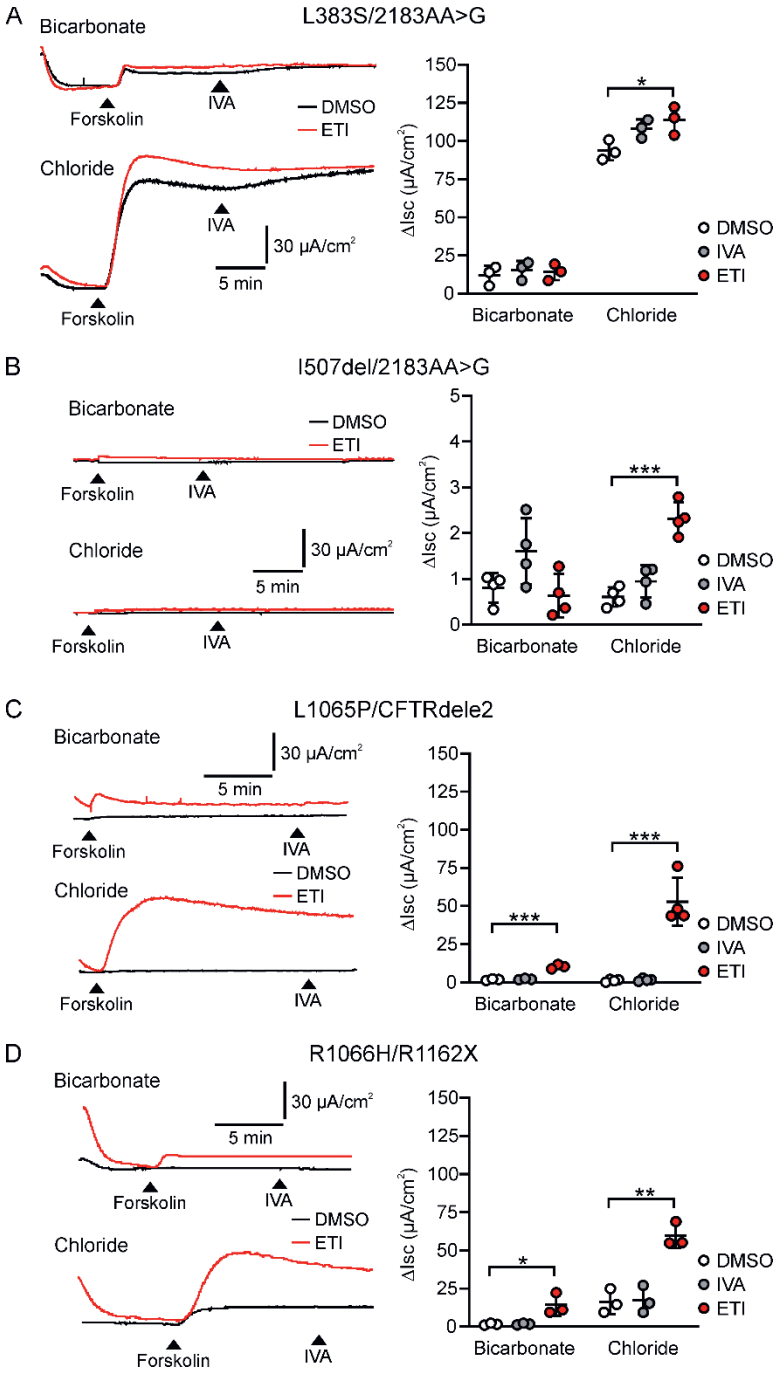
The R1066H genotype showed some residual CFTR activity: Isc responses in chloride and bicarbonate medium amounted to 13% and 11%, respectively, of the responses in WT monolayers. ETI treatment of R1066H-CFTR monolayers resulted in a significant increase in CFTR-mediated chloride and bicarbonate secretion. In contrast, IVA, in the absence of ELX and TEZ, had no effect.

Table 2. Forskolin-dependent Isc responses in CF intestinal epithelial monolayers, in the absence or presence of CFTR modulators.

Genotype [allele 1] [allele 2]	Treatment	Isc Cl- µA/cm2	n	Rel. WT (%)	Isc HCO3- µA/cm2	n	Rel. WT (%)
[WT] [WT]	-	123.9±26.9	6	-	15.2±5.1	6	-
[F508del] [F508del]	ELX/TEZ/IVA	36.0±4.9	4	29	6.9±1.3	4	45
[L383S] [2183AA>G]	DMSO	93.8±6.5	3	76	12.1±6.3	3	80
	IVA	108.2±6.0	3	87	15.4±6.1	3	101
	ELX/TEZ/IVA	113.9±9.2*	3	92	14.1±5.4	3	95
[I507del] [2183AA>G]	DMSO	0.6±0.2	4	0.5	0.8±0.3	4	5.3
	IVA	0.9±0.4	4	0.7	1.6±0.7	4	11
	ELX/TEZ/IVA	2.3±0.4***	4	1.9	0.6±0.5	4	3.9
[L1065P] [CFTRdele2]	DMSO	1.4±0.9	4	1.1	2.0±0.5	3	13
	IVA	1.9±0.9	4	1.5	2.3±0.5	3	15
	ELX/TEZ/IVA	53.1±15.6***	4	43	10.5±1.4***	3	69
[R1066H] [R1162X]	DMSO	16.3±7.9	3	13	1.6±0.6	3	11
	IVA	17.3±9.0	3	14	1.8±0.6	3	12
	ELX/TEZ/IVA	59.6±8.1**	3	48	14.3±7.0*	3	94

Data for F508del- and WT-CFTR are based on organoid cultures derived from one or two tissue donors, respectively; *n* represents the number of technical replicates. * p<0.05; ** p<0.01; *** p<0.001, relative to vehicle (DMSO) control (ANOVA). ELX, elexacaftor; TEZ, tezacaftor; IVA, ivacaftor.

(Next page) Figure 3. Effect of CFTR modulators on the forskolin-dependent Isc in CF epithelial monolayers. The forskolin-dependent Isc was assessed in epithelial monolayers cultured from intestinal organoids. Organoids were prepared from rectal tissue of pwCF with genotypes: (A) L383S/2183AA>G, (B) I507del/2183AA>G, (C) L1065P/CFTRdele2, or (D) R1066H/R1162X. Monolayers treated for 24h with ELX/TEZ or DMSO were bathed in a solution containing either bicarbonate or chloride as the single CFTR permeating anion. Bathing solutions were supplemented with ETI or DMSO as appropriate, and, where indicated, IVA was added to DMSO pre-treated monolayers. Panels to the left show representative experiments, aggregate data of peak Isc responses are shown in the panels to the right. Each data point represents one technical replicate. * p<0.05; ** p<0.01; *** p<0.001, relative to vehicle (DMSO) control (ANOVA).


A
Bicarbonate
L383S/2183AA>G
Forskolin
IVA
DMSO
ETI
Chloride
IVA
30 µA/cm²
5 min
Forskolin
ΔIsc (µA/cm²)
150
125
100
75
50
25
0
*
DMSO
IVA
ETI
Bicarbonate
Chloride
B
I507del/2183AA>G
Bicarbonate
DMSO
ETI
Forskolin
IVA
Chloride
30 µA/cm²
5 min
Forskolin
IVA
ΔIsc (µA/cm²)
5
4
3
2
1
0
***
DMSO
IVA
ETI
Bicarbonate
Chloride
C
L1065P/CFTRdele2
Bicarbonate
30 µA/cm²
5 min
Forskolin
IVA
DMSO
ETI
Chloride
Forskolin
IVA
ΔIsc (µA/cm²)
150
125
100
75
50
25
0
***
***
DMSO
IVA
ETI
Bicarbonate
Chloride
D
R1066H/R1162X
Bicarbonate
30 µA/cm²
5 min
Forskolin
IVA
DMSO
ETI
Chloride
Forskolin
IVA
ΔIsc (µA/cm²)
150
125
100
75
50
25
0
*
**
DMSO
IVA
ETI
Bicarbonate
Chloride

3.5 Effect of elexacaftor/tezacaftor/ivacaftor treatment on forskolin-dependent fluid transport

Because the I507del-, L1065P-, and R1066H-CFTR variants displayed low levels of residual function in our CFTR assay on epithelial monolayers, we performed swelling assays at a forskolin concentration that is projected to lead to optimal phosphorylation/activation of the CFTR pool, and is best suited to detect low levels of functional CFTR (14).

Consistent with the Isc measurements in epithelial monolayers, we observed an ETI-dependent rescue of CFTR function in organoids carrying the L1065P- or R1066H-CFTR variant (Fig. 4). As was observed in epithelial monolayers, IVA alone did not significantly improve the function of these variants. The Isc measurements on the I507del-CFTR variant had indicated a small increase in chloride transport after ETI treatment, but rescue of CFTR function was not apparent in the swelling assay. We speculate that if rescue of CFTR occurs under these conditions, the gain in function is too minute to effect an increase in organoid size over a 1h incubation period.

The L383S-CFTR variant displayed significant forskolin-dependent swelling in the absence of CFTR modulators. Notably, after circa 40 min of forskolin exposure, no further increase in organoid size was detected. Different from the other three lines, organoids expressing L383S-CFTR had accumulated fluid prior to the start of the assay (e.g. see Fig. 1). Because the amount of fluid that can be accrued in a lumen that is enclosed by a given number of epithelial cells is necessarily limited, prior fluid uptake/swelling will constrain the response to subsequent forskolin stimulation. We conjecture that this is why the swelling response of this particular variant reached a plateau. Previously, it was shown that, for similar reasons, the forskolin-induced swelling assay (FIS) underestimates WT-CFTR function (14). It follows that this type of assay is well-suited to assess the response to CFTR modulators of those genotypes which accumulate little fluid in the absence of such compounds, but its interpretation becomes more intricate when variants with progressively more subtle functional defects are evaluated.

Whereas in the Isc measurements we observed a modest increase in CFTR activity after ETI treatment, neither ETI, nor IVA alone, enhanced swelling of L383S-CFTR carrying organoids. ETI even appeared to reduce forskolin-dependent, normalized swelling, when compared to vehicle treated organoids. Conceivably, prolonged (24h), combined ELX and TEZ treatment further stimulated swelling in this genotype, blunting the response to subsequent forskolin stimulation.

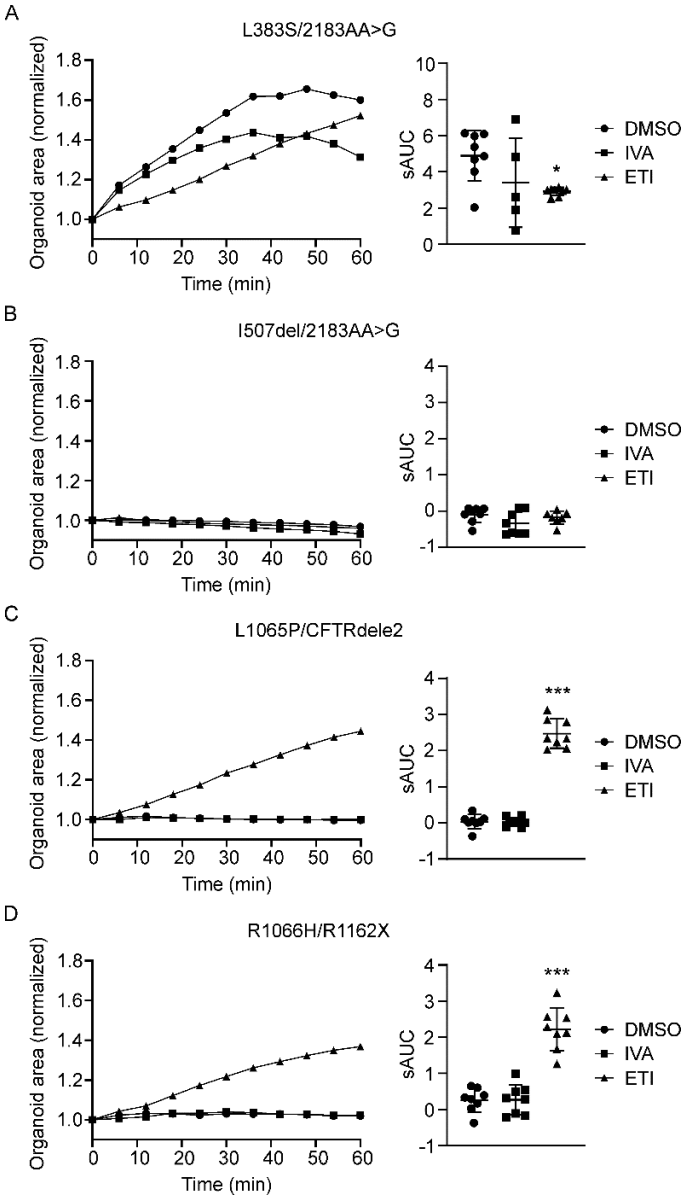


Figure 4. Effect of CFTR modulators on forskolin-dependent organoid swelling. Rectal organoids with genotypes (A) L383S/2183AA>G, (B) I507del/2183AA>G, (C) L1065P/CFTRdele2, or (D) R1066H/R1162X were treated with IVA, ETI, or DMSO (vehicle), and CFTR activity was triggered by addition of forskolin. Time courses (left panels) show the surface area occupied by organoids, relative to the area at the start of the experiment. Data points represent means of 5-8 technical replicates (wells). Panels to the right show the cumulative increase in area over the 60 min period, expressed as the signed area under the curve (sAUC), where negative values denote a decrease in area relative to $t = 0$. Each data point represents one technical replicate. * $p<0.05$; *** $p<0.001$, relative to DMSO (ANOVA).

4. Discussion

Our study aimed at assessing the response to IVA and ETI of the rare CFTR variants L383S, I507del, L1065P or R1066H, expressed in primary intestinal cells stemming from pwCF. Importantly, the individuals who donated these cells carried these poorly characterized CFTR variants *in trans* of a minimal function allele, considered unresponsive to modulators. This enabled us to specifically evaluate the function and rescue of these rare variants. Of these four variants, only R1066H is currently one of the 178 approved for ETI therapy in the U.S. However, it has not yet been approved for treatment of pwCF in most other countries. Unusually, the approval of R1066H (and many other rare variants) by the FDA was not based on clinical trials, but relied on *in vitro* studies, in which CFTR-mediated chloride transport was assessed in FRT cells transfected with the appropriate CFTR variant (8). The organoid model allowed us to assess modulator-dependent restoration of the anion secretory function of these variants in a proper genomic context, in cells natively expressing CFTR.

Our electrophysiological assays demonstrated that CFTR activity in epithelial monolayers prepared from the individuals with I507del/2183AA>G, L1065P/CFTRdele2 or R1066H/R1162X genotypes was low (≤13% of WT-CFTR). Consistent with these functional data, we did not detect fully glycosylated CFTR protein (C-band) in organoids with these genotypes. Moreover, the individuals from which these organoids originated displayed a comparatively severe disease phenotype, typified by pancreatic insufficiency and overt lung disease.

ETI treatment improved processing of L1065P- and R1066H-CFTR, resulting in a marked increase in the abundance of the fully glycosylated protein. Congruently, ETI significantly enhanced CFTR activity in L1065P- and R1066H-CFTR expressing epithelial monolayers. For both variants, the ETI-dependent improvement in chloride-secretory function amounted to >25 % of WT-CFTR. It is plausible that such a marked response translates into a clinical benefit, e.g. it is comparable, or even superior, to the ETI-dependent gain in function of F508del-CFTR measured presently in intestinal cells, and previously in the FRT model (8, 18). Apart from improving CFTR-mediated chloride transport, ETI treatment also enhanced bicarbonate secretion across monolayers expressing these variants, indicating that ETI therapy may counter luminal hyper-acidification and decrease mucus viscosity in intestinal (and, possibly, other) epithelia (2, 3). Patch-clamp experiments have indicated that the CFTR corrector lumacaftor (VX-809) increases the permeability of F508del-CFTR to bicarbonate more than its permeability to chloride (23). A limitation of our study is that, because bicarbonate and chloride transport were measured in intact epithelia, we could not determine the relative permeability of the presently investigated CFTR variants to these anions. Notwithstanding, it is of interest that in L1065P- and R1066H-CFTR epithelial monolayers, as well as in the F508del-CFTR specimens, ETI appeared to restore

bicarbonate transport more effectively than chloride transport (see percentages in Table 2), which is consistent with (but not proof of) a comparable shift in the selectivity of the channel pore of these variants. In contrast to the robust response to ETI of the L1065P and R1066H variants, I507del-CFTR showed only a marginal increase in ETI-dependent anion secretion, and even in the presence of the modulator combination, I507del-CFTR function remained <4% of WT-CFTR.

The function and response to ETI of the I507del, L1065P and R1066H variants has recently been studied in FRT cells (18). As in our present assays, CFTR-dependent chloride (but not bicarbonate) transport across epithelial monolayers was assessed by electrophysiology. This model allows a direct comparison of the functional characteristics and rescue of single CFTR variants in a genetically uniform, albeit nonhuman, cellular background. However, it does not account for inter-individual genetic variability within the *CFTR* locus or other genomic regions that can affect expression and function of CFTR variants, and their response to modulators (25-27). Such genetic variability may partly explain why the clinical response to modulator therapy can differ substantially between carriers of apparently identical *CFTR* alleles (27). Encouragingly, although different models were used, the results of this previous *in vitro* study are consistent with our present results on patient-derived primary cell cultures, both with respect to the low residual activity of these variants, and their response to ETI treatment. This consistency implies that ETI may be efficacious in a wider group of pwCF that carry either the L1065P or R1066H variant. Indeed, recently, ETI was shown to restore CFTR function in intestinal organoids stemming from an individual who carried the R1066H mutation *in trans* of the class I mutation CFTRdele2,3 (14).

To our knowledge, the effect of modulator treatment on processing and activity of the exceedingly rare variant L383S-CFTR has not been studied before. In our assays, L383S-CFTR displayed considerable residual function (>50% of WT), and Western blot analysis suggested that protein synthesis and processing is not notably impaired by this mutation. Congruent with the moderate functional defect *in vitro*, the clinical data of the donor also suggest that some residual CFTR function is retained *in vivo*, i.e. this individual presented with pancreatic sufficiency, and an intermediate sweat test result. However, the clinical presentation also comprised MSSA colonization of the respiratory tract and a markedly lowered FEV1, indicative of a more prominent disease presentation in the lungs. Consequently, CFTR activity in airway epithelium may be affected more than is suggested by the moderate increase in sweat chloride, the absence of overt pancreatic disease, or the high residual CFTR activity presently observed in intestinal cells. A predominant presentation of CF-like disease symptoms in a single organ is commonly observed in those with a *CFTR* genotype of unknown or variable clinical significance (25, 26). It suggests that secondary genetic factors, outside the

CFTR locus, affect expression and/or activity of such variants in a tissue-specific manner (25, 26). We surmise that, in individuals carrying such alleles, the response to modulator treatment may also differ between cell types/tissues (28, 29). Therefore, it is conceivable that ETI treatment is more efficacious in restoring CFTR function in airway epithelium of this particular individual than may be anticipated, based on its modest effect on the anion secretory response in intestinal cells.

Our data indicate that the organoid-based fluid transport assay and the anion transport assay on epithelial monolayers yielded largely consistent results when variants with low residual CFTR activity were evaluated. However, as our analysis of the L383S-CFTR genotype demonstrates, for CFTR variants displaying substantial residual activity, the interpretation of the organoid swelling assay is less straightforward. In this scenario, when intestinal organoids accumulate fluid under basal conditions (prior to the assay), FIS-type tests tend to undervalue CFTR activity, and other organoid-based assays that have an extended dynamic range, such as SLA (steady-state lumen area), or the ICM-type assay used presently, may be more apposite (14, 15).

In conclusion, we have demonstrated that ETI restores the activity of the CF-causing variants L1065P and R1066H. To a more moderate extent, this modulator combination also improved the function of the very rare variant L383S. These findings may guide the development of a personalized medicine approach for the individuals included in this study. Our data align with previous studies on the FRT cell model, presently used to assess the response of rare variants to modulator therapy, and support the use of organoid-based assays to expedite the delivery of efficacious drugs to pwCF carrying (combinations of) rare CFTR variants not previously evaluated.

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Chapter 4

Ivacaftor improves both bicarbonate and chloride transport by the CFTR variant D1152H (c.3453G>C) in primary human intestinal epithelial cells

Dora Angyal[1], Layla Moesker[1], Tessa A. Groeneweg[1], Claudio Sorio[2], Paola Mellotti[3], Jeffrey Beekman[4], Hettie Janssens[5], Marcel J.C. Bijvelds[1]

[1]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands
[2]Department of Medicine, University of Verona, Division of General Pathology, Cystic Fibrosis Laboratory "D. Lissandrini", Verona, Italy
[3]Cystic Fibrosis Centre, Azienda Ospedaliera Universitaria Integrata Verona, Verona, Italy
[4]Department of Pediatric Respiratory Medicine, Wilhelmina Children's Hospital, University Medical Center, Utrecht University, Utrecht, The Netherlands
[5]Department of Pediatrics, Division of Respiratory Medicine and Allergology, Erasmus MC-Sophia Children's Hospital, Erasmus MC University Medical Center, Rotterdam, The Netherlands

In preparation

Part I

Abstract

Introduction: Cystic fibrosis is caused by mutations in the *CFTR* gene, which encodes an epithelial anion channel that conducts both chloride and bicarbonate. The CFTR variant D1152H (c.3453G>C) is typically associated with a mild disease phenotype, and was reported to confer a selective defect in bicarbonate conductance. However, other reports have indicated that it also affects chloride conductance. Here, we measured epithelial chloride and bicarbonate transport across intestinal epithelium derived from D1152H carriers, and tested the effect of the potentiator ivacaftor (IVA) on D1152H-CFTR-mediated anion transport.

Methods: Organoids were generated from rectal biopsies of four compound heterozygotes carrying the D1152H-CFTR allele *in trans* of a minimal function allele (class I mutation). Activation of the WNK/SPAK signaling pathway, thought to increase the permeability of CFTR to bicarbonate, was assessed by Western blot analysis. Epithelial monolayers were prepared from organoids. Forskolin-dependent, CFTR-mediated chloride and bicarbonate secretion across these monolayers was assessed by short-circuit current (Isc) measurement, in bathing media that contained either bicarbonate or chloride as the single CFTR-permeating anion.

Results: Detection of phosphorylated SPAK indicated activation of the WNK/SPAK pathway in intestinal cells. Both chloride and bicarbonate secretion were lowered in D1152H-CFTR expressing monolayers, compared to monolayers expressing wild type (WT) CFTR (for all four: <40%). In D1152H-CFTR monolayers, the Isc response carried by bicarbonate amounted to between 10-20% of the current measured in chloride medium. A similar percentage was observed in monolayers expressing WT-CFTR, and those expressing other gating mutants (A455E, G551D, S1251N). IVA enhanced both D1152H-CFTR-mediated bicarbonate and chloride secretion.

Conclusion: The D1152H mutation, endogenously expressed in intestinal cells, impairs intestinal bicarbonate and chloride secretion to a similar extent, and both bicarbonate and chloride transport improved upon IVA treatment. Because bicarbonate/chloride transport ratios in D1152H-CFTR expressing epithelia were similar as in lines expressing WT-CFTR or other gating mutants, no specific defect in bicarbonate conductance was evident.

Introduction

Cystic fibrosis (CF) is a potentially fatal disease syndrome caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. *CFTR* encodes an epithelial anion channel that mediates both chloride and bicarbonate transport across epithelia, principally located in the respiratory and the gastrointestinal tract. CFTR-mediated anion secretion drives osmotic fluid secretion in ductal structures, and controls the pH at epithelial surfaces. In CF, the acidic luminal pH causes the accretion of an abnormally viscid mucus that obstructs ductal structures, and promotes microbial colonization and inflammation.

It has been put forward that some mutations in the CFTR protein specifically affect its permeability to bicarbonate. In a heterologous expression system (HEK-293T cells), these variants showed apparently normal chloride, but a lowered bicarbonate permeability (1). According to this model, a low extracellular chloride concentration, by activation of the chloride-sensitive protein kinase WNK and its substrate SPAK, leads to a conformational change in CFTR that specifically enhances the permeability of the channel pore to bicarbonate (2). As the experiments on HEK-293T cells demonstrated, the co-expression of these protein kinases was sufficient for triggering a change in CFTR anion selectivity. A group of nine bicarbonate-defective CFTR (CFTR-BD) variants were found more commonly in a cohort of pancreatitis patients than in controls, implying that carrier status confers an increased risk of developing pancreatitis (1).

Among these CFTR-BD variants identified, was the gating mutant D1152H. Congruent with a specific defect in bicarbonate permeability, D1152H-CFTR demonstrated reduced bicarbonate-, but normal chloride transport function in human nasal epithelial cells and HEK 293T cells (1, 3). However, arguing against a specific defect in bicarbonate transport, previous studies have shown that carriers of this allele can present with increased sweat chloride levels, indicative of impaired CFTR-dependent chloride transport in the sweat glands (4). It has also been shown that the D1152H mutation leads to a substantial reduction in chloride transport when studied in monolayers of Fischer Rat Thyroid (FRT) cells transfected with CFTR (5, 6). Consistent with these latter data, we found that not only bicarbonate transport, but also chloride transport was significantly impaired in intestinal epithelium stemming from a subject carrying the D1152H-CFTR variant *in trans* of a minimal function allele (7). Moreover, we observed that treatment with a CFTR modulator combination consisting of elexacaftor, tezacaftor, and ivacaftor improved not only bicarbonate-, but also chloride transport in this experimental model.

Between carriers of the D1152H allele, sweat test results may vary widely, ranging from normal to CF-like. D1152H, combined with a CF allele *in trans* results in a variable disease presentation. Accordingly, carriers may be diagnosed with CF or CFTR-related

disorder (CFTR-RD, e.g. CBAVD, bronchiectasis), but may also be asymptomatic. This suggests that expression of the D1152H-CFTR variant results in across-subject variation in channel activity. The CFTR potentiator ivacaftor (IVA) was shown to enhance chloride secretion across D1152H-CFTR expressing epithelia (5, 6). In nasal epithelium, IVA was shown to enhance CFTR-mediated bicarbonate transport, but it is unclear whether it also stimulates chloride secretion across this epithelium (3).

To assess whether the D1152H-CFTR variant confers a specific defect in epithelial bicarbonate transport, we generated intestinal organoids from rectal biopsies of four individuals carrying D1152H-CFTR. We cultured epithelial monolayers from these organoids, and used a modified ICM (intestinal current measurement) procedure to separately assess either CFTR-mediated bicarbonate or chloride secretion. Intestinal epithelial cells robustly express the WNK and SPAK type protein kinases, which, according to some studies, promote CFTR-mediated bicarbonate transport (1, 2). In all four subjects, D1152H-CFTR was expressed *in trans* of a minimal function allele containing a class I mutation. Consequently, any CFTR activity detected in our assay, and any potential stimulatory effects of IVA observed, must be attributed to the D1152H allele.

Materials and methods

Tissue collection

This study was conducted according to the guidelines in the Declaration of Helsinki and was approved by the local medical ethics committees. Written, informed consent was obtained from all participating subjects. Organoids were included from the laboratories of the Erasmus MC University Medical Center, Rotterdam (MEC 2012-317) and Borgo Roma Hospital, Verona (protocol #CFTR028), as well as the HUB Biobank, Utrecht (MTA 21/034 and MTA 25/054).

Organoid culture

Rectal biopsies, collected for surveillance or diagnostic purposes, were used for culturing organoids as described in detail elsewhere (7). In brief, to dissociate the epithelial cells from the underlying connective tissue, biopsies were incubated in phosphate buffer saline (PBS) supplemented with EDTA (10 mmol/L; Life Technologies) for 20-90 min at 4 °C. Epithelial cell clusters were suspended in extracellular matrix (Matrigel, Corning) and cultured in a medium containing the growth factors EGF, Noggin, R-Spondin 1 and Wnt3a. Media were supplemented with Y27623 and CHIR-99021 (10 µmol/L each; Merck) during the first two days after seeding.

Culture of epithelial monolayers

Epithelial monolayers were generated from organoids as previously described (7). Briefly, after cryopreserved cultures were thawed, they were expanded by sub-cultivation (1:5) over 3-4 cycles. Organoid-derived cells were seeded on filter inserts (250,000/cm^2; Transwell #3470, Costar). The culture medium was identical to the one used for extracellular matrix-embedded organoids. Before seeding, filter inserts were treated with extracellular matrix (Matrigel) diluted in PBS (1:20) for 2h at 37°C. Formation of epithelial monolayers was monitored by measuring of the transepithelial electrical resistance (TEER) using an epithelial volt/ohmmeter (EVOM; World Precision Instruments). Monolayers were cultured for circa 3 weeks, resulting in TEER values typically >200 Ω.cm^2.

Chloride and bicarbonate transport across epithelial monolayers

Filter inserts were mounted in an Ussing chamber (P23023T/P2300, Physiologic Instruments). For assessing CFTR-mediated chloride transport, monolayers were bathed in (mmol/L) 128 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 Na-isethionate, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose. For assessing CFTR-mediated bicarbonate transport, monolayers were bathed in (mmol/L) 128 Na-isethionate, 4.7 K-isethionate, 1.3 $Ca(CH_3COO)_2$, 1.0 $Mg(CH_3COO)_2$, 20 $NaHCO_3$, 0.4 NaH_2PO_2, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose. The only CFTR-permeating anion in this latter solution is

bicarbonate. Bathing solutions were maintained at 37°C and gassed continuously with O2 or 95% O_2/5% CO_2, respectively. The potential difference across epithelial monolayers was set at 0 mV using a VVC-MC8 voltage clamp module (Physiologic Instruments), and short-circuit currents (Isc) were recorded using LabChart 8 software (AD Instruments). After the Isc had reached a steady state, baseline Isc values were recorded. Addition of the cAMP agonist forskolin (10 µmol/L; Bio-Connect BV) was used to activate CFTR channel activity, followed by IVA (0.3 µmol/L; Selleckchem). Experiments were designed to allow for a paired comparison, i.e. monolayers cultured from the same organoid passage, maintained in the same tissue culture plate and culture medium batch, were assayed in parallel to assess the anion secretory response in bicarbonate *vs*. chloride medium.

Western blot analysis

Organoids were cultured for 6 days after seeding, as described above. For assessing the effect of extracellular chloride and bicarbonate on SPAK phosphorylation, organoids were incubated in Meyler solution (mmol/L: 128 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose), or equivalent solutions in which either bicarbonate or chloride was isosmotically substituted by isethionate (15 min, 37°C). Cells were collected and washed in cell recovery solution (Corning) to remove extracellular matrix proteins, and subsequently lysed in NaCl (150 mmol/L), Tris/HCl pH 7.6 (25 mmol/L), Triton X100 (1%), sodium deoxycholate (1%), sodium dodecyl sulfate (0.1%), NaF, (5 mmol/L), Na_3VO_4 (3 mmol/L), supplemented with a protease inhibitor cocktail (HALT; Thermo Fischer Scientific). After the protein concentration was determined using a reagent kit (Bio-Rad), lysates (40 µg) were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membrane. A polyclonal antibody was used that detects SPAK phosphorylated at Ser373, and the phylogenetically related kinase OSR1 phosphorylated at Ser325 (07-2273; Millipore). Detection of ß-actin served as a reference (Sc-47778; Santa Cruz). Primary antibodies were detected using fluorescently labeled secondary antibodies (goat-anti-mouse- or goat anti-rabbit IgG; Invitrogen #A21057 and #A32735). Fluorescence was detected and analyzed using an Odyssey imaging system and associated software (Image Studio Lite 5.2; Licor Biosciences).

Statistical analysis

Data are expressed as means ± S.D. As specified in the figure legends, the Student t-test or ANOVA were used to determine the statistical significance of differences between means (SPSS 28.0, IBM). Differences were considered statistically significant when $P < 0.05$.

Results

Activation of WNK and SPAK in D1152H-CFTR intestinal organoids

CFTR-mediated bicarbonate transport was shown to be dependent on the activity of WNK/SPAK signal transduction route (1, 2). WNK-type protein kinases are activated by a lowering of cellular chloride, which leads to phosphorylation of the WNK substrate SPAK (8). We assessed the effect of extracellular chloride on SPAK phosphorylation (which is indicative of WNK activation) in intestinal organoids by Western blot analysis. We detected phosphorylated SPAK in all four lines analyzed (Fig. 1). In all four lines, removal of chloride from the incubation medium increased the level of phosphorylated SPAK.

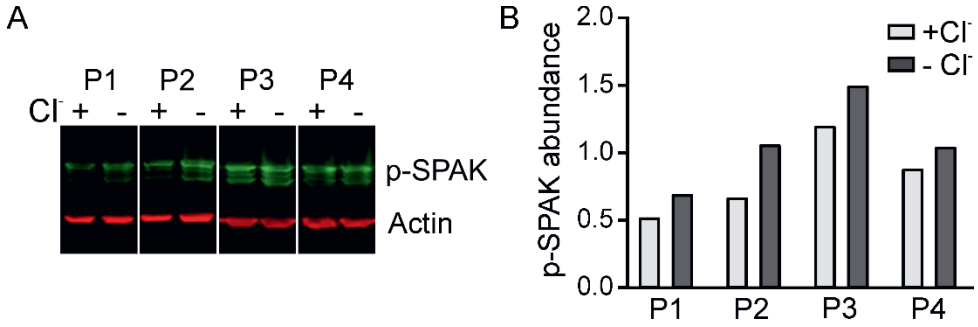


Figure 1. Phosphorylation of SPAK was detected by Western blot analysis in lysates prepared from intestinal organoids from subjects P1-P4. Organoids were incubated (15 min) in the presence or absence of extracellular chloride. (A) Phosphorylated SPAK (p-SPAK) was detected by Western blot analysis. Detection of ß-actin served as a loading control. (B) p-SPAK abundance in the presence or absence of extracellular chloride, normalized to ß-actin abundance.

The D1152H mutation impairs chloride and bicarbonate transport across intestinal epithelium

The protocol normally used for performing ICM (intestinal current measurement) on rectal biopsies was adapted to assess CFTR-mediated transport across epithelial monolayers that were cultured from intestinal (rectal) organoids. Forskolin-stimulated, CFTR-dependent currents were measured in bathing media that contained either chloride or bicarbonate as the single CFTR-permeating anion. Removal of extracellular chloride is also projected to activate the WNK-type protein kinases that may control the anion selectivity of the CFTR channel pore (2). We observed that the forskolin-dependent currents in all four lines, both in chloride and bicarbonate medium, were substantially lower than those detected in monolayers expressing WT-CFTR (Table 1) (9). Notwithstanding, all lines showed considerable residual channel activity, in both chloride and bicarbonate medium, commensurate with the often comparatively mild phenotype of D1152H carriers.

Table 1. Forskolin-dependent Isc responses in CF intestinal epithelial monolayers.

Subject	Genotype	Isc HCO_3^-			Isc Cl^-		
	[allele 1] [allele 2]	µA/cm2	*n*	Rel. WT (%)	µA/cm2	*n*	Rel. WT (%)
	[WT] [WT]	10.1±2.2	4	-	105.5±8.9	4	-
P1	[D1152H] [R553x]	5.4±1.0***	4	53	44.4±18.2***	4	42
P2	[D1152H] [L732X]	3.6±0.3***	4	36	27.0±7.9***	4	26
P3	[D1152H] [R1162X]	5.9±1.9**	4	58	43.7±11.7***	4	41
P4	[D1152H] [3905insT]	2.2±0.4***	5	22	15.9±2.4***	5	15

n represents the number of technical replicates. **P<0.01; ***P<0.001, relative to WT. Statistical analysis: one-way ANOVA

To assess whether bicarbonate transport is specifically impaired by the D1152H mutation, we determined the Isc response in bicarbonate medium relative to the response in chloride medium for these four lines, and compared these with the corresponding ratios measured in WT-CFTR expressing monolayers, and those expressing other CFTR variants that exhibit residual channel function, but are not suspected of conferring a specific defect in bicarbonate permeability. We observed that ratios were comparable between all lines tested, and no specific impairment in bicarbonate transport in the D1152H lines was indicated (Fig. 2).

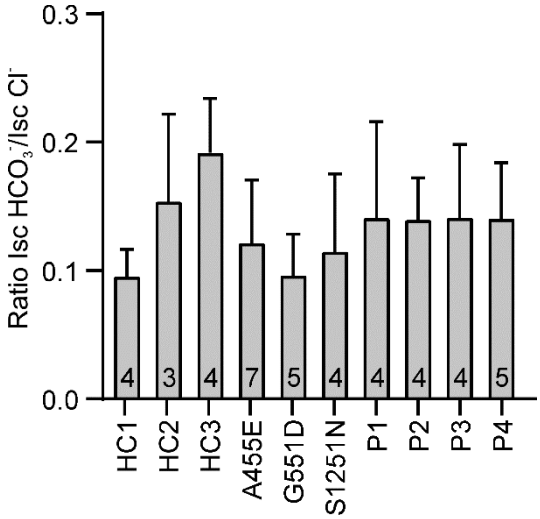


Figure 2. Ratio of the forskolin-dependent Isc response of intestinal epithelial monolayers in bicarbonate- *vs.* chloride-medium of three wild type CFTR expressing subjects (HC1-HC3), subjects expressing the indicated CFTR variants (*in trans* of a F508del allele), and subjects P1-P4. The number of technical replicates is as indicated in the bars.

IVA enhances D1152H-mediated bicarbonate and chloride transport across intestinal epithelium

IVA treatment acutely stimulated D1152H-CFTR mediated chloride and bicarbonate transport across monolayers of all four lines (Fig. 3). This is consistent with previous studies showing that the function of the D1152H allele is amenable to rescue by CFTR potentiators, including IVA (5, 6). Because it has been reported that some CFTR modulators may change the anion selectivity of the CFTR channel pore, we assessed the ratio between forskolin-dependent bicarbonate vs. chloride currents, in the presence or absence of IVA (10, 11). We observed that IVA increased this bicarbonate/chloride ratio in three out of four D1152H-CFTR lines tested, i.e. in these lines (P1, P3 and P4) the relative increase in bicarbonate transport elicited by IVA was larger than the rise in chloride transport (Fig. 4). However, in P2, no consistent increase in this ratio was observed.

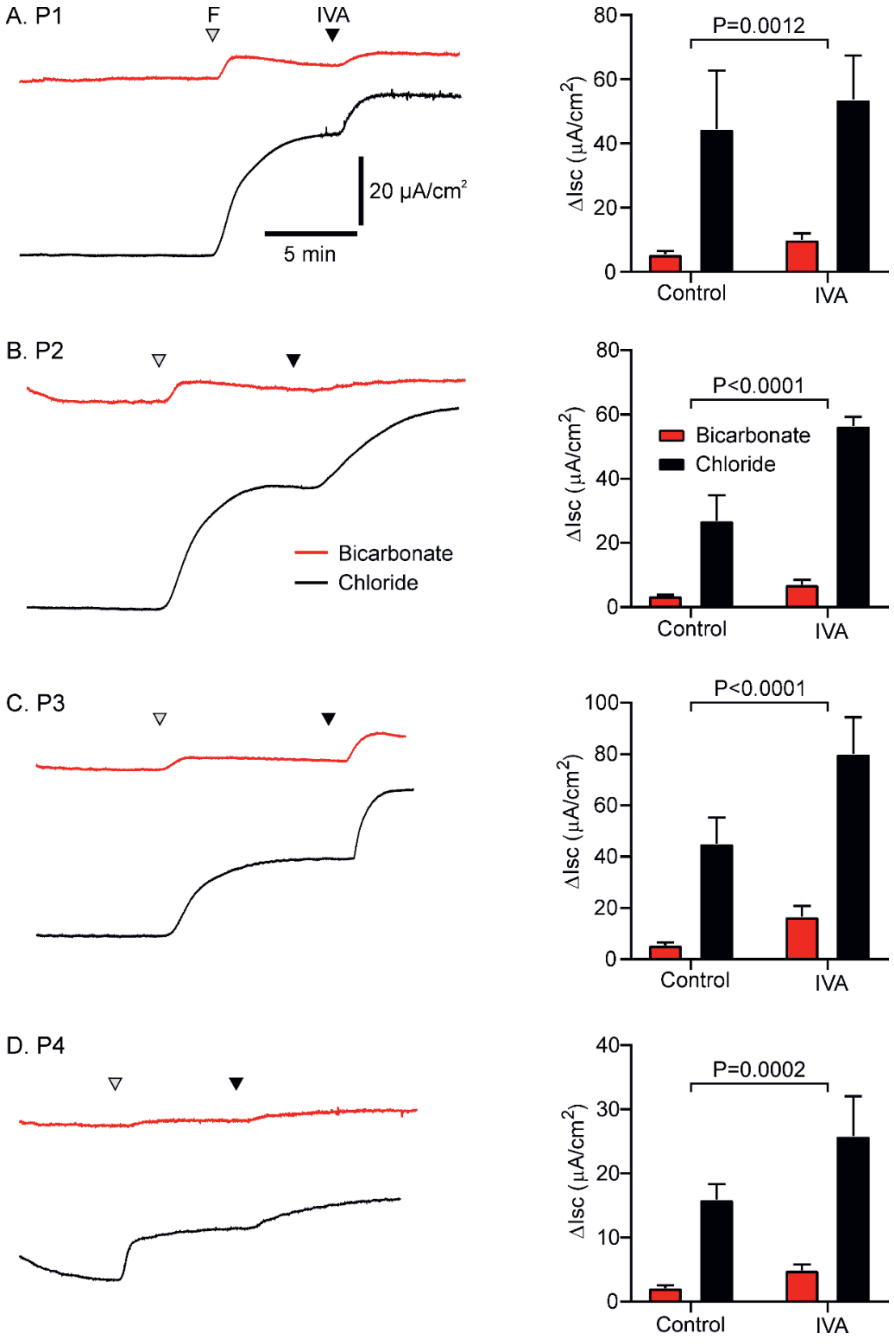


Figure 3. Effect of IVA on CFTR-mediated bicarbonate and chloride transport across intestinal epithelial monolayers of P1-P4. (A-D) Left panels show representative recordings. Where indicated, forskolin (F) was added to stimulate CFTR activity, followed by IVA. Bar charts represent aggregate data. Technical replicates: 4 (P1, P2, P3) or 5 (P4). Statistical analysis: two-way ANOVA.

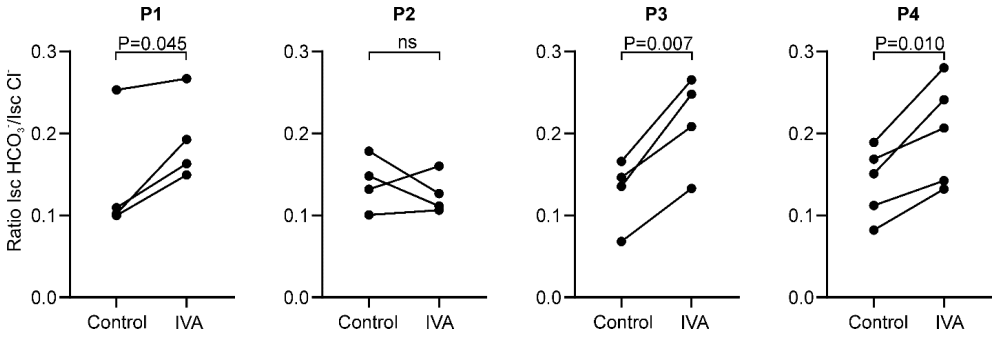


Figure 4. Effect of IVA on the ratio of the forskolin-dependent Isc response in bicarbonate- vs. chloride- medium. Each data point represents one technical replicate. Statistical analysis: paired t test.

Discussion

In this study, we demonstrate that the D1152H mutation significantly reduces chloride and bicarbonate secretion across intestinal epithelium. Still, the bicarbonate/chloride transport ratios measured in all four lines were similar to the corresponding ratio in WT-CFTR expressing monolayers and in other CFTR variants displaying residual function. IVA treatment enhanced both chloride and bicarbonate secretion across D1152H-CFTR monolayers. Our data demonstrate that, because it impairs CFTR-mediated chloride secretion to a similar extent as bicarbonate secretion, the D1152H mutation does not confer a specific defect in bicarbonate permeability.

Our data are consistent with previous reports showing a 20-50% reduction in chloride transport in FRT monolayers transfected with D1152H-CFTR (5, 6). Similar as in our approach, these FRT assays were performed in a bathing medium containing only chloride as the CFTR-permeating anion, but, consequently, did not evaluate the bicarbonate transport function of D1152H-CFTR. Similar applies for swelling assays performed on intestinal organoids. While these showed that IVA improved the response of D1152H-CFTR, they are typically performed in a bicarbonate-buffered, chloride-rich cell culture medium, and do not allow separate evaluation of the CFTR bicarbonate transport function (12, 13).

Different from our present work on intestinal epithelia, a previous study has shown that forskolin-dependent bicarbonate, but not chloride, transport across D1152H-CFTR-expressing nasal epithelium was impaired (3). These contrasting observations suggest that the anion transport properties of D1152H-CFTR vary between different tissues, perhaps reflecting differences in regulation by the WNK pathway. WNK-type protein kinases regulate epithelial chloride and bicarbonate secretion in various tissues, principally by controlling the cell surface expression of the ion transport mechanisms involved (8). This tissue-specific control of protein localization extends to the

basolateral ion transport mechanisms required for cellular chloride- and bicarbonate uptake from the interstitial fluid. It is conceivable that, in nasal epithelium, these are rate limiting for transepithelial chloride secretion, not only in epithelia expressing WT-CFTR, but also in epithelia expressing CFTR variants that exhibit a substantial residual channel function, such as D1152H-CFTR. In this scenario, such epithelia expressing CFTR variants with a reduced chloride conductance may display apparently normal rates of transepithelial chloride secretion. In contrast, because cellular uptake of bicarbonate relies on the activity of other transporters, in epithelia expressing a CFTR variant with a reduced anion conductance, bicarbonate secretion may be limited by apical efflux.

Further, we cannot exclude the possibility that considerable variation exists in the expression of epithelial anion transport mechanisms between individual carriers of a D1152H allele. The presently used organoid cultures were collected from those that presented with CF-like clinical symptoms. In this cohort, the loss of CFTR-mediated chloride- as well as bicarbonate transport function may have predisposed to the development of this relatively severe phenotype. One may conjecture that bicarbonate-specific defects are more readily detected in D1152H-CFTR carriers diagnosed with a CFTR-RD, like chronic pancreatitis (1). Considering that CFTR has a critical role in pancreatic, ductal bicarbonate secretion, it is perhaps unexpected that all 15 homozygous carriers of the D1152H-CFTR allele registered in the CFTR2 database have a pancreatic sufficient status, and only 23% of all D1152H-CFTR carriers were registered as pancreatic insufficient (www.cftr2.org, accessed June 20, 2025). This suggests that the D1152H mutation does not severely compromise pancreatic CFTR function.

We observed that D1152H-CFTR-mediated bicarbonate and chloride transport are enhanced by IVA treatment, in line with beneficial effects seen in vitro and in patients (12). In three subjects, IVA treatment increased the bicarbonate/chloride transport ratios. Because no such effect was observed in the fourth subject, this is unlikely to be a direct effect of IVA on the relative anion permeability of the CFTR channel pore. Rather, this inter-subject variation points to the role of other factors that control/limit transepithelial, CFTR-dependent anion secretion, like a variable expression of the aforementioned ion transport mechanism mediating cellular anion uptake. Nevertheless, previous work has indicated that some CFTR modulators increase the permeability of F508del-CFTR to bicarbonate, but the response of other CFTR variants, or the effect of IVA was not investigated (10, 11). Because we measured chloride and bicarbonate transport across intact epithelia, and, therefore, did not directly assess the permeability of CFTR to anions, we cannot entirely exclude the possibility that IVA modulates the relative anion permeability of D1152H-CFTR (10, 11).

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Chapter 5

CFTR function is impaired in a subset of patients with pancreatitis carrying rare *CFTR* variants

Dora Angyal[1*], Karina Kleinfelder[2*], Fabiana Ciciriello[3], Tessa A. Groeneweg[1], Giulia De Marchi[4], Nicolò de Pretis[4], Laura Bernardoni[4], Luca Rodella[5], Francesco Tomba[5], Paola De Angelis[6], Cecilia Surace[7], Emily Pintani[8] Federico Alghisi[3], Hugo R. de Jonge[1]#, Paola Melotti[8], Claudio Sorio[2], Vincenzina Lucidi[3], Marcel J.C. Bijvelds[1], Luca Frulloni[4]
*Equal contribution. #Deceased.

[1]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
[2]Department of Medicine, University of Verona, Division of General Pathology, Verona, Italy
[3]Cystic Fibrosis Unit, Bambino Gesù Children's Hospital, IRCCS, Piazza di Sant'Onofrio 4, 00165 Rome, Italy
[4]Gastroenterology Unit, Department of Medicine, Borgo Roma Hospital, Piazzale L.A. Scuro 10, 37134 Verona, Italy
[5]Endoscopy Surgery Unit, Azienda Ospedaliera Universitaria Integrata Verona, 37126 Verona, Italy.
[6]Digestive Endoscopy and Surgery Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy
[7]Laboratory of Medical Genetics, Bambino Gesù Children's Hospital, IRCCS, Viale di San Paolo 15, 00146, Rome, Italy
[8]Cystic Fibrosis Centre, Azienda Ospedaliera Universitaria Integrata Verona, Verona, Italy.

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Part I

Abstract

Background: Many affected by pancreatitis harbor rare variants of the cystic fibrosis (CF) gene, *CFTR*, which encodes an epithelial chloride/bicarbonate channel. We investigated CFTR function and the effect of CFTR modulator drugs in pancreatitis patients carrying *CFTR* variants.

Methods: Next-generation sequencing was performed to identify *CFTR* variants. Sweat tests and nasal potential difference (NPD) assays were performed to assess CFTR function *in vivo*. Intestinal current measurement (ICM) was performed on rectal biopsies. Patient-derived intestinal epithelial monolayers were used to evaluate chloride and bicarbonate transport and the effects of a CFTR modulator combination: elexacaftor, tezacaftor and ivacaftor (ETI).

Results: Of 32 pancreatitis patients carrying *CFTR* variants, three had CF-causing mutations on both alleles and yielded CF-typical sweat test, NPD and ICM results. Fourteen subjects showed a more modest elevation in sweat chloride levels, including three that were provisionally diagnosed with CF. ICM indicated impaired CFTR function in nine out of 17 non-CF subjects tested. This group of nine included five carrying a wild type *CFTR* allele. In epithelial monolayers, a reduction in CFTR-dependent chloride transport was found in six out of 14 subjects tested, whereas bicarbonate secretion was reduced in only one individual. In epithelial monolayers of four of these six subjects, ETI improved CFTR function.

Conclusions: CFTR function is impaired in a subset of pancreatitis patients carrying *CFTR* variants. Mutations outside the *CFTR* locus may contribute to the anion transport defect. Bioassays on patient-derived intestinal tissue and organoids can be used to detect such defects and to assess the effect of CFTR modulators.

Keywords: cystic fibrosis; epithelial cells; organoids; pancreatic ducts

1. Introduction

Pancreatitis is an inflammatory disorder of the acinar and ductal epithelia of the exocrine pancreas. It causes considerable discomfort and suffering and is among the most common gastrointestinal diagnoses associated with hospital admission (1). Conventionally, inflammation is thought to be initiated by the premature conversion of the zymogen trypsinogen, produced by acinar cells, into the active protease. This inappropriate activation of proteolysis within the pancreas itself leads to tissue injury, which, in turn, triggers immune cell infiltration. Inflammation may be contained to the pancreas, but in severe cases a systemic response may ensue that can culminate in multiple organ failure (2, 3). Acute pancreatitis may resolve spontaneously, but if the underlying causes driving tissue inflammation are not adequately addressed, episodes of acute pancreatitis can recur, or inflammation may become chronic (4-6).

Multiple genetic and environmental factors contribute to the etiology of pancreatitis (3, 7-9). In cases where no obvious environmental or metabolic trigger (e.g. alcohol abuse, hyperlipidemia, biliary disease, autoimmunity) is suspected, it is often challenging to identify the cause of acute (recurrent) or chronic pancreatitis (6). However, mutation of one of several genes thought to be involved in the intra-pancreatic activation of trypsin (IPAT) has been shown to confer an increased risk of pancreatitis (7-9). One such IPAT gene is *CFTR*, which encodes a phosphorylation-regulated anion channel that mediates chloride and bicarbonate transport across various epithelia (10).

Loss-of-function mutations in *CFTR* on both alleles cause cystic fibrosis (CF), a complex multisystem disorder characterized by loss of anion and fluid secretion across epithelia of the respiratory and gastrointestinal tract, including that of the intestine, and the biliary and pancreatic ductal tree. In pancreatic ducts, CFTR-dependent bicarbonate and osmotic fluid secretion drives the rapid passage (flushing) of digestive enzymes to the intestine (11, 12). In CF patients carrying minimal function mutations, obstruction of the pancreatic ducts with viscid mucus and protein aggregates can already develop *in utero*, leading to exocrine pancreatic insufficiency at an early stage of life (13). Only those who carry *CFTR* mutations that permit residual anion channel function may preserve adequate exocrine pancreatic function. Still, these pancreatic sufficient CF subjects are more likely to develop pancreatitis (14). *CFTR* variants leading to a more moderate impairment in CFTR function do not cause CF, but are associated with several CFTR-related disorders (CFTR-RD), often with a predominant presentation in a single organ. Typical manifestations of CFTR-RD are bronchiectasis, congenital bilateral absence of the vas deferens, and pancreatitis (15, 16).

It has been estimated that up to 30% of those with idiopathic pancreatitis carry mutations in the *CFTR* gene (17, 18). These numbers suggest a critical role of CFTR-dependent ductal anion and fluid secretion in containing the deleterious effects of

pathogenic stimuli. In fact, several typical stressors (e.g. bile acids, ethanol, trypsin) were shown to directly impact ductal CFTR function (19-21). The consequent reduction in ductal bicarbonate and fluid secretion is thought to precipitate ductal cell injury and inflammation (22). These findings not only imply that impairment of CFTR function is crucial in the development of pancreatitis, but also suggest that, for some forms, loss of ductal CFTR function initiates pancreatitis, and precedes acinar pathology (3, 22, 23).

While the detection of *CFTR* variants is suggestive of CFTR-RD, in many of these cases, the functional and clinical consequences of these mutations are uncertain, and further diagnostic procedures to evaluate CFTR function are indicated. This has become even more pertinent now that CFTR modulators, i.e. small-molecule compounds that can restore the function of some (but not all) *CFTR* variants, have become clinically available (24). CFTR modulators were shown to improve biomarkers of exocrine pancreas function in CF patients and animal models, and to ameliorate disease in an experimental model of autoimmune pancreatitis characterized by acquired CFTR dysfunction (23, 25-27). These data indicate that CFTR modulators may be applicable for treatment of those forms of pancreatitis in which CFTR dysfunction is suspected.

In this study we sought to assess CFTR function and the potential beneficial effects of CFTR modulator therapy in a group of subjects diagnosed with idiopathic pancreatitis, carrying mostly rare, poorly characterized *CFTR* variants. To evaluate CFTR function in this cohort, in addition to performing sweat tests and nasal potential difference (NPD) measurements, we used transepithelial current measurements to assess CFTR-mediated chloride and bicarbonate transport separately. Because pancreatic tissue cannot routinely be sampled from patients and no validated method is currently available that allows direct assessment of CFTR function in the pancreatic ductal epithelium, we used intestinal tissue and organoid-derived epithelial monolayers to evaluate the impact of mutations on CFTR-mediated anion transport. Intestinal organoids have been routinely used for assessing the response to modulators of *CFTR* variants in the context of CF research, and CFTR assays on intestinal organoids were shown to correlate with CFTR function in patient tissue (28, 29). Like the cells lining the pancreatic ductal tree, intestinal epithelial cells robustly express the WNK- and SPAK-type protein kinases, which, according to some studies, are required (and sufficient) for promoting CFTR-mediated bicarbonate secretion (30-32). We considered CFTR-mediated bicarbonate transport of particular interest because mutations that were reported to interfere with the regulation of the bicarbonate permeability of CFTR by the WNK/SPAK pathway, were proposed to increase the risk of pancreatitis (31, 33). Furthermore, generation of organoid-derived intestinal epithelial monolayers allowed us to evaluate the effect of a CFTR modulator combination consisting of elexacaftor (ELX), tezacaftor (TEZ) and ivacaftor (IVA; when combined: ETI) on both CFTR-mediated bicarbonate and chloride transport.

2. Methods

2.1 Selection of patients

Patients were enrolled between July 2015 and June 2021 in two centers: the Cystic Fibrosis Unit of Bambino Gesù Children's Hospital, IRCCS and the Gastroenterology Unit, Department of Medicine of Borgo Roma Hospital, Verona. Inclusion criteria were: patient affected by acute, recurrent (≥2 episodes), or chronic pancreatitis, age ≥6 years (4, 34). Exclusion criteria were: pregnancy, solid organ or hematological transplantation, pancreatitis caused by autoimmune disease, gallstones, or hypertriglyceridemia, paraduodenal pancreatitis, consumption of >1 alcohol unit per day. Subjects were screened for *CFTR* mutations by targeted next-generation sequencing (see *Genetic Analysis* below), and included if at least one mutation was detected. Subjects were diagnosed with CFTR-RD, based on established diagnostic guidelines (35). All participating subjects (or their legal representative) signed an informed consent document. The study was approved by the ethical committee of Bambino Gesù Children's Hospital, IRCCS (protocol #2183/2020), and the Borgo Roma Hospital, Verona (protocol #CFTR028).

2.2 Genetic analysis

Targeted Next-Generation Sequencing (NGS) was employed to sequence a panel of 9 IPAT (intra-pancreatic activation of trypsin) genes associated with pancreatitis: *CFTR* (NM_000492.3), *SPINK1* (NM_003122.3), *PRSS1* (NM_002769.4), *PRSS2* (NM_002770.2), *CTRC* (NM_007272.2), *CASR* (NM_001178065.1), *CTSB* (NM_147780.2), *CPA1* (NM_001868.2) and *KRT8* (NM_002273) (7, 9). Gene sequences were obtained through the Genome Browser online platform (https://genome.ucsc.edu/). Libraries were prepared using Twist Custom Panels (Twist Bioscience) and NGS was performed on a NovaSeq 6000 instrument (Illumina). Detected variants were identified using the Geneyx analysis platform (Geneyx) and were validated by Sanger sequencing. For all patients, the *CFTR* poly-T tract and TG tract were analyzed. Variants 7T and 9T in combination with any TG variant are reported as wild type (WT).

2.3 In vivo CFTR function measurements

Gibson and Cooke sweat test (GCST) was performed as routine procedure within the framework of regular patient care, according to established protocols (36). Mean sweat chloride levels <30 mmol/L were considered within the normal range, whereas levels ≥60 mmol/L are considered pathognomonic for CF (37). The bubble sweat test (BST) was performed according to a previously published protocol (38, 39). Briefly, the cholinergic agonist methacholine (M) and a cocktail (C) of β adrenergic agonists mixed with atropine were applied intradermally to stimulate CFTR-independent and -

dependent sweating, respectively. C/M ratios ≥0.205 are considered to indicate normal CFTR function, while CF patients typically show ratios ≤0.0055. Nasal potential difference (NPD) measurement was performed according to the standard operating procedure of the European Cystic Fibrosis Society (ECFS) (40). For discriminating CF patients, a locally validated Wilschanski Index (WI) cut-off value of 0.82 was used (41).

2.4 Intestinal organoid and epithelial monolayer culture

Organoids were generated from rectal forceps biopsies and maintained in medium containing the growth factors Wnt3a, Noggin, R-Spondin 1 and EGF in Matrigel matrix (Corning), according to established protocols (42). For culture of epithelial monolayers, organoids were suspended in advanced DMEM (4°C; Gibco) and washed by centrifugation (5 min, 400 g) to remove the extracellular matrix. Cells were dissociated by brief (45 sec, 37°C) incubation in trypsin solution (0.25%; Gibco), followed by repeated aspiration through a 200 µL pipette tip (Greiner). Trypsin activity was quenched by addition of fetal calf serum (10%) in advanced DMEM, and cells were washed in advanced DMEM and filtered through a cell strainer (70 µm; Falcon). Cells were collected by centrifugation and seeded (6.10^5 cells/cm^2) on permeable inserts (Transwell #3470; Corning) pretreated with diluted Matrigel (1:20 in phosphate buffered saline, 0.2 mL/cm^2, 2h, 37°C). Culture medium was as for organoids, except that CHIR99021 (10 µmol/L; Sigma-Aldrich) and Y-27632 (10 µmol/L; R&D Systems) were added during the first 2 days after seeding. Cells were cultured until a confluent monolayer was obtained (10-14 days), which was monitored by measuring the transepithelial electrical resistance using an EVOM2 device (World Precision Instruments).

2.5 Electrophysiological assessment of epithelial anion transport

Ion transport in rectal biopsies was assessed according to standard operating procedures for intestinal current measurement (ICM; SOP v.2.7; https://www.ecfs.eu/ctn/standardization-committees) (40, 43). After washing in ice-cold phosphate buffered saline, biopsies were mounted on P2407C type inserts (surface area: 0.011 cm^2; Physiologic Instruments), in low-volume chambers (P2250; Physiologic Instruments). Tissue was bathed in Meyler solution (mmol/L: 128 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4) supplemented with indomethacin (10 µmol/L), HEPES (10 mmol/L) and glucose (10 mmol/L), maintained at 37°C, and gassed with 95% O_2, 5% CO_2. The transepithelial potential difference was clamped at 0 mV (EVC4000 module; World Precision Instruments), and the resulting short-circuit current (Isc) was recorded with a PowerLab 8/35 AD-converter and associated software (LabChart 8; AD Instruments). After a 10 min equilibration period, amiloride (100 µmol/L) was added to the luminal bathing medium to inhibit ENaC-mediated Na^+ currents. After a further 10 min period, the cAMP agonists forskolin (10

µmol/L) and 3-isobutyl-1-methylxanthine (IBMX; 100 µmol/L) were added to stimulate CFTR activity, followed by carbachol (100 µmol/L) and histamine (500 µmol/L). Cumulative Isc response to forskolin/IBMX, carbachol and histamine were determined as described elsewhere (40, 43, 44). Data shown represent the mean response of two biopsy specimens per subject.

Organoid-derived intestinal epithelial monolayers were incubated with ELX (VX-445, 3 µmol/L; MedChem), TEZ (VX-661, 3 µmol/L; SelleckChem) and IVA (VX-770; 0.3 µmol/L; SelleckChem), or vehicle (DMSO, 0.2%) for 20h. Subsequently, filters were mounted in P2302T/P2300-type Ussing chambers (surface area: 0.33 cm^2; Physiologic Instruments). Monolayers were initially bathed in Meyler solution supplemented with HEPES (10 mmol/L) and glucose (10 mmol/L). For assessing CFTR-dependent chloride transport, a similarly formulated solution was used, except that $NaHCO_3$ was replaced by Na-isethionate. The pH of this solution was set at 7.35 by NaOH titration. For assessing CFTR-dependent bicarbonate transport, NaCl and KCl were replaced by the isethionate salts, and $CaCl_2$ and $MgCl_2$ by acetic acid salts of these cations. The only CFTR-permeating anions in these solutions are chloride and bicarbonate, respectively. Where applicable, CFTR-modulator drugs were re-added. Solutions were maintained at 37°C, gassed with 95% O_2, 5% CO_2 or, in case a bicarbonate-free solution was used, O_2. The transepithelial potential difference was clamped at 0 mV (VVC-MC8 module; Physiologic Instruments), and the resulting short-circuit current (Isc) was recorded as for ICM. Forskolin (10 µmol/L) was used to stimulate CFTR activity. CFTR-dependence of the forskolin-dependent Isc response was verified by addition of the CFTR inhibitor CFTRinh172 (20 µmol/L; Tocris Bioscience). Data shown represent the forskolin-dependent, CFTRinh172-sensitive change in Isc.

2.6 Statistical analysis

The statistical significance of mean differences in anion transport between epithelial monolayers and controls, and the effect of CFTR modulators on anion transport was evaluated by ANOVA, using Dunnett's test to correct for multiple comparisons (SPSS v.28; IBM).

3. Results

3.1 CFTR variants detected in pancreatitis patients

Thirty two subjects diagnosed with idiopathic pancreatitis were enrolled (Table 1). The age of subjects at the onset of pancreatitis ranged between 3 and 49 years. In 18 (56%) patients abdominal imaging revealed abnormalities: pancreatic duct dilatation (6, 19%) and pancreatic calcifications (4, 13%) were reported most frequently, and pancreas divisum was reported in one patient (S.32). Endoscopic retrograde cholangiopancreatography (ERCP) was performed on 22 (69%) patients: 16 (50%) underwent sphincterotomy and four (13%) recurrent stent placement. Two patients reported steatorrhea (S.14, S.18). These and three additional patients (S.01, S.03, S.11) had fecal elastase-1 concentrations below 100 µg/g indicating severe exocrine pancreas dysfunction, while two patients (S.10, S.25) had fecal elastase-1 concentrations between 100 and 200 µg/g indicating mild to moderate exocrine pancreas dysfunction. In one of these patients (S.11), the hemoglobin A1c (HbA1c) level was elevated, suggestive of endocrine pancreatic dysfunction. One patient, S.01, had a lowered forced expiratory volume in one second (FEV_1) of 48%, indicative of pulmonary disease.

Table 1. Demographics and clinical characteristics of patients. A total of 32 subjects diagnosed with idiopathic pancreatitis was enrolled. CF: cystic fibrosis, CFTR-RD: CFTR-related disorder; CP: chronic pancreatitis, RAP: recurrent acute pancreatitis associated with *CFTR* variant, Provisional: final diagnosis pending further assessment.

Demographic		**Assessed, n**
Females, n (%)	16 (50%)	32
Age at inclusion, mean (range)	26 (7-54)	32
Age at onset of pancreatitis, mean (range)	21 (3-49)	32
Positive family history, n (%)	5 (16%)	32
Clinical characteristics		
ERCP, n (%)	22 (68.8%)	32
Abdominal imaging abnormalities, n (%)	18 (56.3%)	32
Other GI symptoms present, n (%)	4 (12.5%)	32
Fecal elastase-1 <200 µg/g, n (%)	7 (25.0%)	28
HbA1c >40 mmol/mol, n (%)	1 (3.8%)	26
FEV_1 <70%, n (%)	1 (6.7%)	15
Lung colonization, n (%)	9 (28.1%)	32
Diagnosis, n (%)	CF: 3 (9.4%) CFTR-RD: 4 (12.5%) CP: 5 (15.6%) RAP: 13 (40.6%) Provisional: 7 (21.9%)	32

Twelve (38%) patients carried CF-causing variants and 27 (84%) carried alleles with varying or unknown clinical consequences (Table 2) (45, 46). Among these, seven (22%) carried a *CFTR* variant that previously was classified as specifically defective in bicarbonate transport (CFTR-BD), namely R75Q (S.21), I148T (S.08) L997F (S.15, S.16, S.25), D1152H (S.05) and D1270N (S.24) (31, 33). Sixteen (50%) patients carried one *CFTR* allele on which no mutations were detected. To evaluate the complex genetic risk for pancreatitis, patients were tested for the presence of mutations in other genes involved in idiopathic pancreatitis (*SPINK1, PRSS1, PRSS2, CTRC, CASR, CTSB, CPA1* and *KRT8*) (7, 9). Eleven (34%) patients carried mutations in one of these genes, and one patient (S.14) carried mutations in both *CTRC* and *SPINK1* (Table 2). Three patients were eventually diagnosed with CF (S.01, S.02, S.05), and three additional patients, all displaying colonization of the respiratory tract by pathogenic bacteria, were provisionally diagnosed with CF (S.03, S.04, S.06; Table 2). Pending further CFTR functional assays to exclude CFTR-RD, three patients were provisionally diagnosed with chronic pancreatitis (S.13, S.14, S.16).

All four subjects diagnosed with CFTR-RD were compound heterozygous, carrying one or two *CFTR* variants of varying clinical consequence (Table 2). These individuals had developed chronic pancreatitis, but showed no signs of CF-typical lung disease (e.g. lung colonization and/or lowered FEV1). Of the eight other subjects (provisionally) diagnosed with chronic pancreatitis, seven carried a mutation in an IPAT gene, which, plausibly, had contributed to disease progression. Among these, S.18 carried a disease causing mutation in *PRSS1* (R122H), that is strongly associated with hereditary pancreatitis (47). In all these subjects diagnosed with chronic pancreatitis, imaging revealed tissue lesions such as pancreatic duct dilation or calcification, but only two individuals in this group had thus far developed pancreatic insufficiency (S.14, S.18). Most (9/13) subjects diagnosed with recurrent acute pancreatitis carried a WT CFTR allele *in trans* of a variant of varying clinical consequence, or a variant known not to cause CF (45, 46). Mutation of an IPAT gene was detected in 3 out of 13 subjects diagnosed with recurrent acute pancreatitis.

Subject	Diagnosis	CFTR genotype		IPAT genotype
		Allele 1	Allele 2	
S.01	CF	**F508del**	**G85E**	-
S.02	CF	**F508del**	5T;TG13	-
S.03	CF*	**3849+10kbC->T**	621+3A->G	CTRC: D190G
S.04	CF*	**3849+10kbC->T**	621+3A->G	CTRC: D190G
S.05	CF	**L732X**	D1152H	-
S.06	CF*	S158T	2752-15C->G	-
S.07	CFTR-RD	**F508del**	5T;TG12	-
S.08	CFTR-RD	*I148T*	G1069R	-
S.09	CFTR-RD	*G576A*	R668C/I991M	-
S.10	CFTR-RD	G194V	*R1162L*	-
S.11	CP	**2183AA->G**	F1074L	PRSS1: E79K
S.12	CP	**2183AA->G**	WT	SPINK1: A56L
S.13	CP*	**R1162X**	WT	-
S.14	CP*	**G542X**	WT	CTRC: G217S; SPINK1: N34S
S.15	CP	*L997F*	WT	SPINK1: N34S
S.16	CP*	*L997F*	WT	CTRC: N78Q, FsX44
S.17	CP	5T;TG12	WT	CASR: V149I
S.18	CP	*R668C*	*G576A*	PRSS1: R122H
S.19	RAP*	**2183AA->G**	3500-57C->T	-
S.20	RAP	**2789+5G->A**	WT	-
S.21	RAP	*R75Q*	F1052V	-
S.22	RAP	V1379I	4095+63T->C	-
S.23	RAP	R74W	V855M	-
S.24	RAP	D1270N	WT	SPINK1: N34S
S.25	RAP	*L997F*	WT	-
S.26	RAP	c.-812T->G	WT	-
S.27	RAP	S1426F	WT	SPINK1: N34S
S.28	RAP	4347+13A->G	WT	-
S.29	RAP	G1069R	WT	-
S.30	RAP	G1069R	WT	-
S.31	RAP	*R31C*	WT	CTRC: E225A
S.32	RAP	*5T;TG11*	WT	-

Table 2. Patient genotype, diagnosis and CFTR function measurements. Subjects were coded, based on (provisional) diagnosis and *CFTR* genotype. CF: cystic fibrosis; CFTR-RD: CFTR-related disorder; CP: chronic pancreatitis; RAP: recurrent acute pancreatitis. *Provisional diagnosis pending further assessment. *CFTR* alleles shown in bold typeface are considered to cause CF when combined with another CF-causing allele (45, 46). WT: wild type allele. Alleles shown in italics do *not* cause CF. Other alleles have variable or unknown consequences; those

GCST	**BST**	**NPD**	**ICM**	**Monolayers**		**Pancreatitis**
[Cl^-] mmol/L	C/M ratio	Wilschanski Index	Isc $\mu A/cm^2$	Isc Cl^- (n) $\mu A/cm^2$	Isc HCO_3^- (n) $\mu A/cm^2$	Age at onset Years
125	**0.0**	**32.1**	**4**			24
84	**0.0**	**1.77**	**9**			20
35	0.21	0.58	**63**			15
32	1.42	0.10	**69**			24
59				**27±8** (4)	**4±1** (3)	5
43	0.56	0.51	248			31
39				26±1 (3)	14±12 (3)	9
36	0.31	0.38	298			23
27				113±6 (3)	13±4 (4)	4
52				93±12 (4)	16±3 (4)	17
31	**0.09**	0.24	**129**	65±16 (3)	13±1 (3)	18
30	**0.07**	**34.3**	**41**	140±8 (4)	16±2 (4)	38
28						5
19						10
29			181	120±42 (5)	19±8 (4)	20
18						9
33				119±11 (4)	14±1 (3)	7
12						3
12		**1.72**	221			45
30	0.21	0.10	178			8
31	0.32	0.32	**123**			30
23	0.21	0.37	**116**	**57±25** (5)	18±6 (5)	36
35	1.04	0.21	**113**	103±38 (4)	30±13 (4)	41
24	0.99	0.33	**93**	179±4 (2)	16±8 (6)	13
28	0.31	3.10	173			21
40	0.40	**1.04**	257			9
11	**0.15**	0.01	**110**			27
37	3.33	0.20	194	120±5 (4)	16±4 (4)	20
20	**0.09**	0.18		**29±10** (7)	9±2 (7)	21
18	0.82	0.08	**70**	**26±10** (7)	11±5 (7)	36
25	0.39	0.08	212			31
15	0.25	0.09	**93**			49

underlined were previously categorized as CFTR-BD variants (31, 33). For GCST, median values are shown when multiple assays were performed. Anomalous, pathological test results are depicted in bold face. For GCST, BST and NPD, empirical threshold values were based on previously reported data (see *Methods*, section 2.3). For ICM, cut-off values were obtained from a cohort of 10 *CFTR* WT individuals (207±68 $\mu A/cm^2$, CI: 159-255). Isc responses in monolayers are shown as mean ± SD.

3.2 Many subjects carrying CFTR variants present with intermediate sweat chloride levels

Whereas sweat chloride levels ≥60 mmol/L are considered pathognomonic for CF, intermediate values in the range of 30 to 59 mmol/L, while not fulfilling diagnostic criteria for CF, are nevertheless indicative of a reduced CFTR function (15). Sweat chloride levels were obtained for all subjects included (Fig. 1). Three patients had sweat chloride concentrations typical of CF. Subject S.01 carried the common CF causing F508del mutation in combination with G85E, another CF-causing mutation (45, 46). S.02 combined the 5T;TG13 haplotype *in trans* of the F508del allele, which combination is also thought to be pathogenic and cause CF (48). S.05 also carried a potentially pathogenic allele combination (L732X/D1152H), and presented with sweat chloride levels ranging between 31 (at age 4) and 79 mmol/L (at age 6). Based on genotype and clinical presentation, these subjects were retrospectively diagnosed with CF. In addition, three patients presenting with an intermediate GCST result and evidence of lung pathology where provisionally diagnosed with CF (S.03, S.04, S.06). Of the remaining 26 subjects, 11 had intermediate sweat chloride levels. Interestingly, whereas most of these were *CFTR* compound heterozygous, in five of these patients no second mutation was found (S.12, S.17, S.20, S.26, S.28). The remaining 11 subjects carrying WT alleles had normal sweat test results.

CFTR-dependent and -independent sweat secretion rates (BST) and NPD were examined in 21 and 22 subjects, respectively (Fig. 1B, 1C). Consistent with GCST, two patients diagnosed with CF (S.01, S.02), showed a strongly reduced level of CFTR-dependent sweat secretion (no BST was performed on the third CF patient, S.05). The majority of non-CF individuals in which BST was assessed, presented with normal secretion ratios (C/M ratio >0.205); only four additional patients (S.11, S.12, S.27, S.29) had sweat secretion ratios in the range typically encountered in heterozygotes carrying a minimal function allele (0.0055-0.205). NPD values were elevated in six patients (S.01, S.02, S.12, S.19, S.25, S.26), two of which were diagnosed with CF (S.01, S.02; no NPD was performed on patient S.05). Of the other four patients with a high NPD result, S.12 and S.26 yielded an intermediate GCST value, and S.12 also showed a lowered BST result.

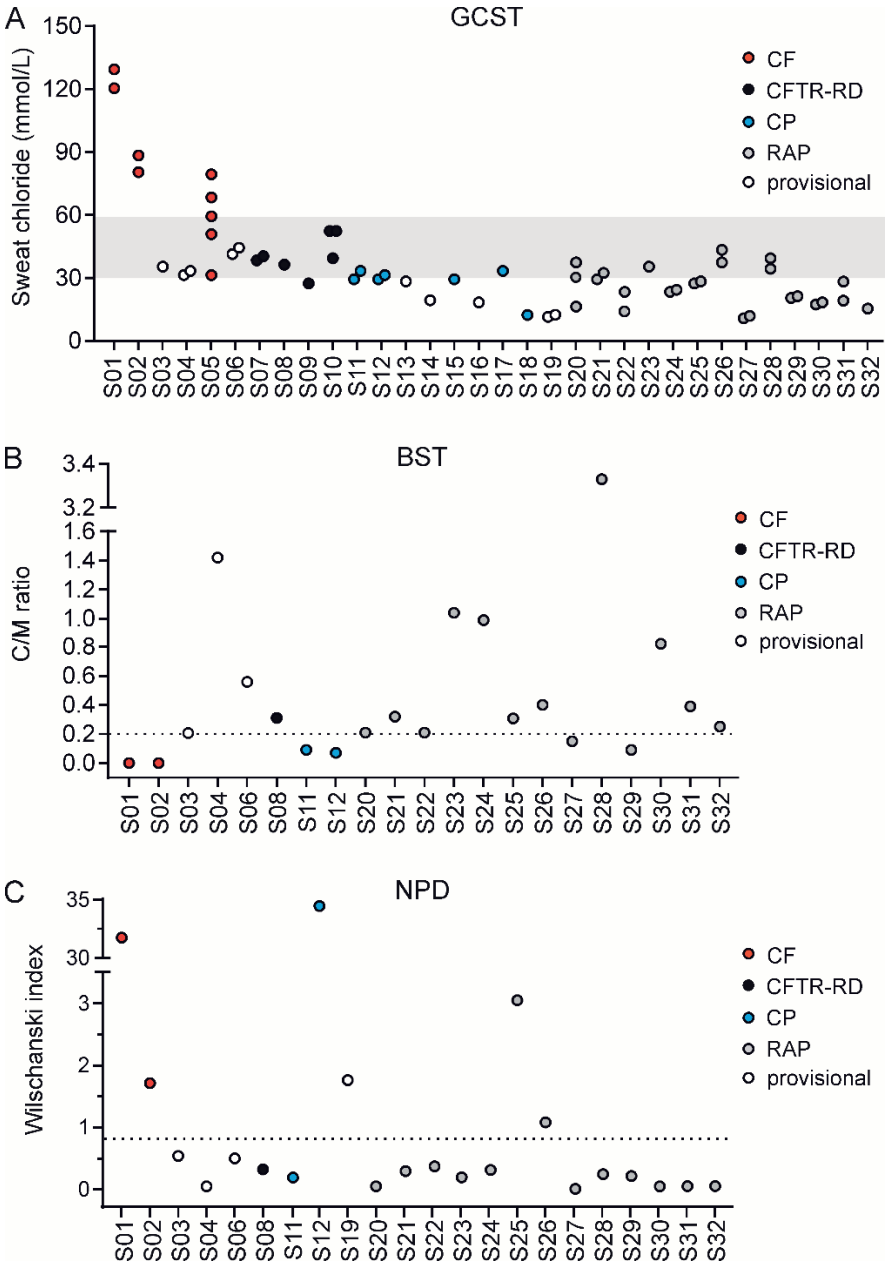


Figure 1. In vivo CFTR function measurements. (A) Gibson and Cooke sweat test (GCST). Data were collected of 32 subjects. Shaded area indicates range of intermediate sweat chloride levels (30-59 mmol/L). Each data point represents one technical replicate. (B) Optical ratiometric sweat test (BST). The ratio of the CFTR-dependent (C) and CFTR-independent (M) rate of sweat secretion was determined in 21 subjects. Dashed line indicates the lower limit for normal values. (C) Nasal potential difference (NPD) measurement. Data were collected of 22 subjects. Dashed line indicates the upper limit for normal values.

3.3 Many subjects carrying CFTR variants show impaired CFTR-mediated intestinal ion transport

CFTR-mediated chloride and bicarbonate secretion was measured in rectal biopsies derived from 22 individuals in this cohort (Fig. 2). CFTR-dependent anion secretion was quantified as the cumulative, net Isc response elicited by forskolin, IBMX, carbachol and histamine. Established procedures for performing ICM were applied, as stipulated by the ECFS (40, 43, 44). For comparison, we also performed ICM in a control cohort of 10 individuals that do not carry mutations in *CFTR*. ICM readily identified the anion secretory defects in S.01 and S.02, the two CF patients already singled out by sweat tests and NPD (no ICM data is available for the third CF patient identified, S.05). Of the remaining 20 subjects, 11 (55%) showed mildly impaired Isc responses, i.e. below the 95% confidence interval (CI) of the present control cohort, but higher than reported for CF subjects (Fig. 2) (43).

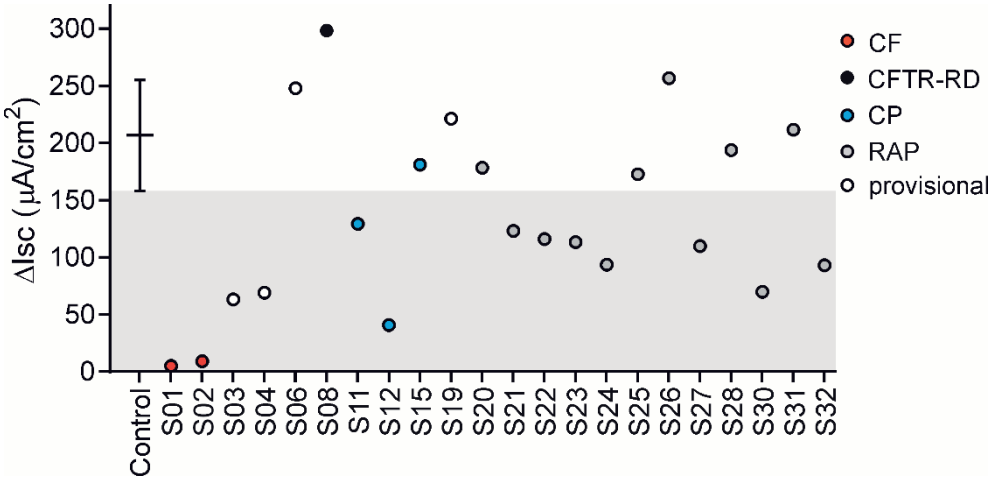


Figure 2. CFTR function measurement in rectal biopsies by ICM. The cumulative Isc response upon forskolin, IBMX, carbachol and histamine administration was assessed in 22 subjects. Data points represent the mean of two technical replicates. For comparison, control values (*CFTR* wild type) are plotted as mean with 95% CI (n=10). Shaded area denotes values <95% CI of the control group.

3.4 CFTR-mediated anion secretion across epithelial monolayers is impaired in a subset of patients

Intestinal monolayers were established from organoids cultured of biopsy specimens obtained from 14 subjects within this cohort. Previous studies have indicated that *CFTR* variants associated with pancreatitis may specifically impair CFTR-mediated bicarbonate transport (31). To evaluate if any of the current subjects carry such variants, separate measurements of chloride and bicarbonate transport were performed. The forskolin-dependent, CFTRinh172-sensitive change in current reflects CFTR-mediated transepithelial chloride or bicarbonate secretion. Five patients (S.05, S.07, S.22, S.29, S.30) showed significantly impaired chloride secretion as compared to non-CF monolayers (Fig. 3A). In addition, monolayers of S.11 also tended to have low chloride secretory responses. In all three patients in this group for which data is available (S.11, S.22, S.30), ICM also indicated lowered anion secretory responses. Results of ICM and current measurements on monolayers corresponded for six out of a total of eight patients for which data of both assays was available. However, S.23 and S.24, while showing moderately lowered CFTR activity in biopsies, showed normal anion secretory responses *in vitro* in cultured cells. For patients S.05, S.07 and S.29 no ICM data was available. However, both S.05 and S.07, but not S.29, had produced intermediate or CF-like GCST results (Fig. 1, Table S1).

Of the epithelial monolayers investigated, three derived from patients carrying *CFTR* variants previously classified as defective in bicarbonate transport (S.05, S.15, S.24). Of these, only monolayers of S.05 displayed impaired bicarbonate secretion (as well as chloride secretion; Fig. 3B). In contrast, S.15 and S.24 showed normal anion secretory responses when assayed in either chloride- or bicarbonate-free solutions.

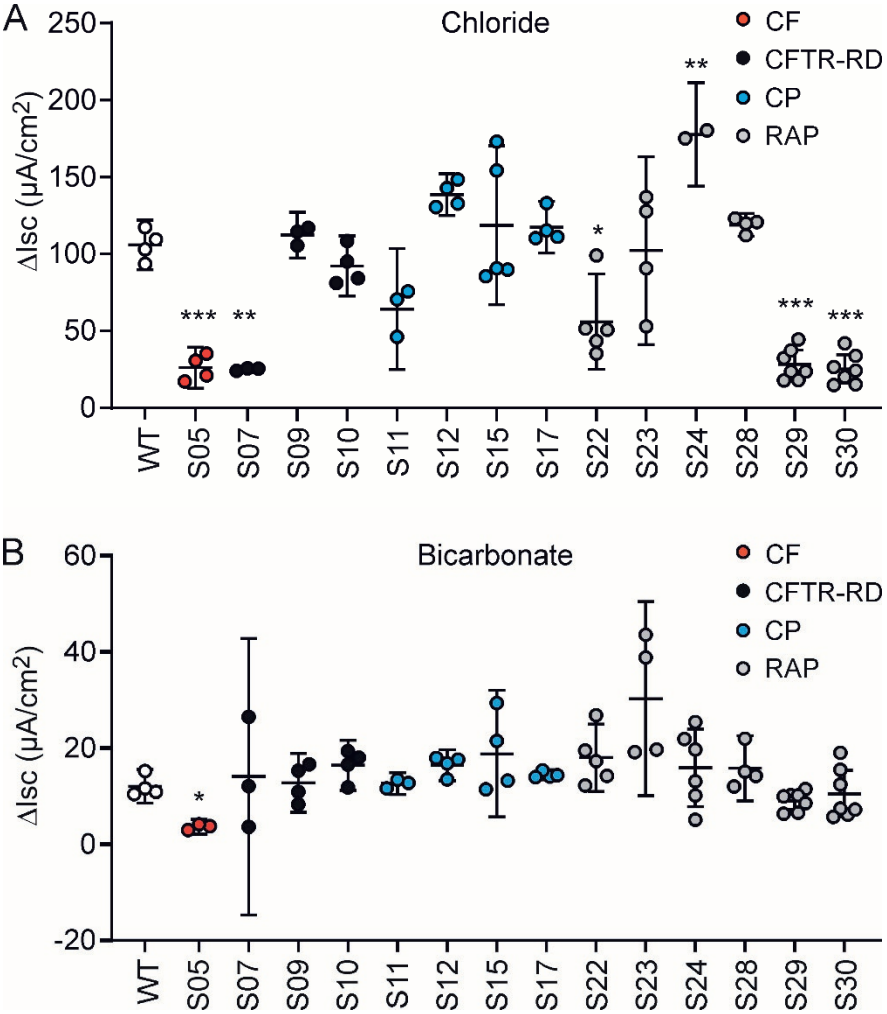


Figure 3. CFTR-dependent chloride and bicarbonate transport in intestinal epithelial monolayers. (A) Forskolin-dependent Isc responses in chloride solution. (B) Forskolin-dependent Isc responses in bicarbonate solution. Data depict mean with 95% CI. Each data point represents one technical replicate. * P<0.05, ** P<0.01, *** P<0.001.

3.5 CFTR-modulators improve anion secretion in some pancreatitis patients

The intestinal organoid model allows evaluation of the effects of CFTR modulators on CFTR function. It is particularly suitable to test compounds that improve CFTR protein maturation and plasma membrane insertion (i.e. correctors), which may take several hours to take full effect. Because of the limited viability of native tissue once removed from the body, such assays are impracticable in intestinal biopsies. The effect of IVA alone or ETI was evaluated in monolayers derived from six patients; those five which demonstrated lowered chloride transport in epithelial monolayers (S.05, S.07, S.22,

S.29, S.30), and in patient S.11 who tended to a lowered response (see Fig. 3). IVA as well as ETI improved chloride transport in monolayers of S.05 (L732X/D1152H), but no significant improvement was observed in the other five patients (Fig. 4A). Both IVA or ETI treatment increased bicarbonate transport in patient S.05. ETI treatment enhanced bicarbonate transport in three additional patients (S.22, S.29, S.30). These results indicate that, in some patients, these modulators selectively improve CFTR-mediated epithelial bicarbonate secretion.

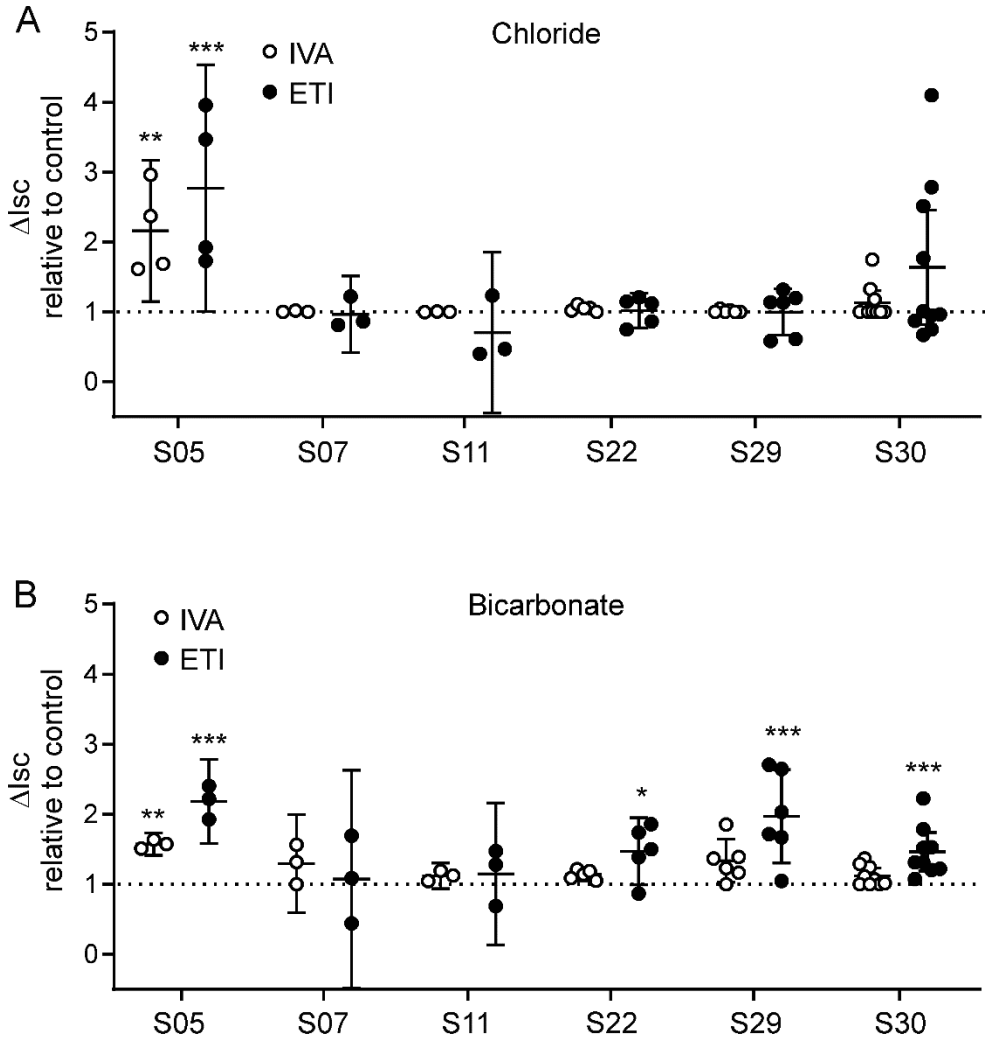


Figure 4. Pharmacological correction of CFTR function assessed on intestinal monolayers. The effect of IVA or ETI treatment on forskolin-dependent, CFTR-mediated chloride (A) and bicarbonate (B) transport. Data depict Isc responses (mean with 95% CI) relative to a paired vehicle (DMSO)-treated control. Each data point represents one technical replicate. * P<0.05, ** P<0.01, ***P<0.001.

4. Discussion

In this study, we investigated CFTR function in 32 individuals affected by recurrent acute, or chronic pancreatitis, carrying mutations in the *CFTR* gene that were mostly of unknown or variable clinical consequence. When such gene variants, suggestive of CF or CFTR-RD, are found, usually a sweat test is performed to evaluate CFTR function. However, mutations that cause only mild or moderate impairment of CFTR function frequently yield sweat test results that are inconclusive, and additional functional tests like NPD and/or ICM are used to further aid diagnosis (37, 44). Presently, we observed that GCST, BST, NPD and ICM yielded consistent data in cases where subjects carry minimal function mutations in *CFTR*, on both alleles. Accordingly, these individuals were retrospectively diagnosed with CF. In contrast, for the other *CFTR* genotypes in this cohort, the outcomes of these tests were often inconsistent. For instance, only three out of ten individuals with intermediate GCST results, presented with abnormal BST and/or NPD measurements. Similarly, in five patients with low responses in ICM, GCST results were in the normal range. These observations align with previous studies showing that in patients carrying alleles of variable clinical consequence, either *in trans* of a CF-causing or a WT allele, CFTR function, as assessed in established bioassays, may vary considerably at different points in time and between tissues (29, 44). Consequently, it is crucial that multiple diagnostic procedures are used to evaluate CFTR function in pancreatitis patients carrying *CFTR* variants. To further improve the utility of these diagnostic tools, a refinement of the criteria for CFTR dysfunction is warranted (15, 37, 40).

ICM indicated that CFTR-dependent anion transport was mildly impaired in 11 out of 22 individuals tested within this cohort, i.e. responses were below normal values, but not to the extent of CF patients carrying two minimal function alleles (e.g. S.01), who typically have negligible ICM responses (40, 43). In five of these subjects (S.12, S.24, S.27, S.30, S.32), next-generation sequencing, which covered not only exons but also the promoter and intronic regions of the *CFTR* gene, identified mutations on only a single allele. This is remarkable because obligate heterozygotes (CF carriers) typically display normal intestinal, CFTR-dependent anion secretion (43, 49). However, consistent with these results, it has been shown before that individuals carrying *CFTR* variants of varying clinical consequence can show considerable temporal and spatial variation in ICM responses, even when they also carry a WT allele. Not only did the ICM response of separate biopsies obtained from such subjects vary more than in wild types, but responses also tended to fluctuate between different points in time (44). In addition, in some, CFTR function appeared to differ in intestinal *vs.* respiratory epithelia, i.e. they displayed CF-typical ICM responses, but NPD results in the normal range (or vice versa). This pattern was mirrored by the clinical manifestations in both organ systems, and low ICM responses were associated with gastrointestinal symptoms. Notably, it was found

that all individuals in this particular cohort that were affected by pancreatitis, showed low CFTR activity in ICM (44). It may be surmised that individuals in this category harbor mutations in separate genomic regions, not covered by the present sequencing protocol, that affect CFTR-dependent epithelial anion secretion in a tissue-specific manner. Conceivably, such "hidden" genetic modifiers affect the transcription of *CFTR* (variants), which is controlled by a combination of locally acting stimuli and transcription factors that often are particular for a given tissue (and cell type). At present, little is known about the transcriptional regulation of variant *CFTR* alleles in the pancreas, and its effects on ductal CFTR function are mostly uncharted. This implies that bioassays performed on other tissues (e.g. NPD, GCST, ICM) must be interpreted with some caution.

Among the currently available techniques ICM is one of a few capable of measuring CFTR function, *ex vivo*, in patient tissue. However, because only a small number of biopsies can be collected, and because ICM must be performed on freshly collected patient material, it may be challenging to perform sufficient replicate measurements to control for potential biological variation between tissue specimens (40, 44). To some extent, this limitation can be addressed by performing current measurements on intestinal organoids or organoid-derived epithelial monolayers. Other than ICM, the organoid model permits numerous measurements to be performed without time constraints, including evaluation of the effects of CFTR modulators on CFTR function at a personal level.

We succeeded in culturing intestinal organoid-derived monolayers from intestinal biopsies obtained from 14 individuals in this cohort. Measurement of CFTR-mediated anion secretion in these epithelial monolayers indicated that chloride transport was impaired in six patients out of these 14 tested. Remarkably, two subjects with low responses carried a *CFTR* WT allele (S.29, S.30), whereas another heterozygote (S.12) generated responses in the normal range. This latter individual carried a minimal function (frameshift) mutation, demonstrating that one functional (WT) allele suffices for a normal anion secretory response. As already indicated by ICM, this suggests that heterozygous subjects with lowered anion secretory responses harbor mutations outside the genetic regions covered by our present sequencing protocol, that affect epithelial anion secretion.

For eight subjects, both ICM and current measurements on monolayers were performed. For six of these, both assays yielded corresponding results. This is consistent with previous data showing that cAMP-dependent anion secretory responses in donor-matched rectal tissue and organoid-derived monolayers correlate, and that these responses are principally, if not fully, dependent on CFTR (40, 50). Notwithstanding, subjects S.23 and S.24 showed low responses in ICM but apparently normal responses *in vitro*, in monolayers. The cause of this disparity between

measurements on rectal biopsies and epithelial monolayers made thereof is speculative. Conceivably, locally acting environmental cues modulate the anion secretory response in intestinal tissue of individuals carrying *CFTR* variants associated with CFTR-RD (44). *In vitro*, in intestinal epithelial organoid cultures, such factors produced by other cell types are lost, which may explain why assays on epithelial monolayers do not necessarily mirror the results attained with ICM. This divergence emphasizes the relevance of preclinical CFTR assays in near native tissues (biopsies), to predict the impact of rare mutations on CFTR function in individuals affected by pancreatitis, CFTR-RD, or mild CF.

Only epithelial monolayers obtained from CF patient S.05 displayed impaired bicarbonate transport. This patient carried the CFTR-BD variant D1152H *in trans* of a minimal function allele (L732X). In contrast, other subjects carrying CFTR-BD variants (S.15, S.24) showed normal bicarbonate and chloride secretory responses, plausibly because they also carried a WT allele. Although categorized as a CFTR-BD variant, chloride secretion was also markedly reduced in S.05. Diagnosed with CF at the age of six based on a pathologic sweat test, this individual suffered two episodes of pancreatitis within 12 months. However, so far, no respiratory symptoms were observed. In line with our present results, it was previously shown that the D1152H mutation not only impairs bicarbonate conductance, but also leads to a substantial reduction (>40%) in CFTR-mediated chloride transport (51). Previous studies have also shown that carriers of this allele can present with an intermediate GCST result and a BST result in the CF range, indicative of impaired chloride transport in the sweat glands (52). In contrast, D1152H-CFTR demonstrated normal chloride transport function in human nasal epithelial cells and a heterologous expression system (HEK 293T cells), suggesting that a single *CFTR* variant can result in across-tissue, and perhaps across-subject, variation in residual CFTR activity (31, 53).

Congruent with previous studies on the D1152H-CFTR variant, the intestinal epithelial monolayers derived from CF patient S.05 strongly responded to modulator treatment (51). Either IVA or ETI treatment not only enhanced bicarbonate transport, but also significantly improved chloride secretion. Indeed, in some countries (including the U.S.), CF patients carrying this variant are eligible for modulator therapy. Importantly, in addition, three other individuals, two of which carried a WT allele, and all diagnosed with recurrent acute pancreatitis, showed a modest improvement in bicarbonate transport upon ETI treatment. Unexpectedly, although ETI enhanced bicarbonate secretion in this group of subjects, it did not improve chloride transport. This suggests that the rate of chloride secretion by intestinal epithelial cells in these individuals is limited by factors other than CFTR, such as the activity of the transporter(s) mediating cellular chloride uptake across the basolateral plasma membrane. Indeed, this is also

indicated by the observation that S.29 and S.30, even though they carry a WT allele, demonstrate low responses in ICM, unlike typical CF carriers (43, 49).

An important limitation of this study is that we did not directly evaluate CFTR function in pancreatic tissue, and we cannot exclude that the effect of the presently investigated mutations on CFTR function differs between pancreatic ductal and intestinal cells, or that the response of the ductal cells to modulator treatment is different from intestinal cells. However, it has been shown that intestinal organoid-based assays correlate well with the efficacy of modulator therapy *in vivo*, and there is accruing evidence that modulator therapy improves pancreatic pathology in CF patients (26, 27, 54-57). The increase in bicarbonate secretion elicited by ETI presently observed, therefore suggests that modulator therapy may improve ductal anion secretion in a select group of individuals affected by pancreatitis. A further limitation is that only a relatively small number of subjects with diverse genotypes was included in this study. The response to modulators, both *in vitro* and *in vivo*, may vary considerably between individuals with apparently identical *CFTR* genotypes (28, 58). Consequently, the effects of modulators observed in individual patients are not necessarily representative for a particular *CFTR* genotype, and drug responses may need to be evaluated on a personal basis, in patient-derived tissues or cells (15, 44). We surmise that, of the currently available techniques to assess CFTR function *in vivo* or in patient tissue, ICM is most suited to evaluate CFTR function in subjects affected by a variant of pancreatitis that is, potentially, associated with CFTR dysfunction. Subsequently, organoids generated from intestinal biopsies can be used to assess the response to CFTR modulators. Once cryopreserved, patient-derived organoids can henceforth be used to test novel drugs when they become available. Ultimately, clinical trials are warranted to establish which of these (compound) *CFTR* genotypes, responsive *in vitro*, truly benefit from modulator therapy.

5. Conclusion

In conclusion, our findings demonstrate that CFTR dysfunction in pancreatitis patients carrying *CFTR* variants is relatively prevalent, indicative of the critical role of CFTR dysfunction in the etiology of pancreatitis (18-23). Impaired CFTR function was also observed in some carrying a WT *CFTR* coding sequence on one allele, suggesting that disease-causing mutations may also be located outside the *CFTR* locus. We show that patient-derived organoids may be used to assess the impact of *CFTR* genotype on epithelial bicarbonate and chloride transport, and the response to CFTR modulator therapy. Further, our data suggest that modulator therapy may be beneficial for carriers of *CFTR* variants that suffer from pancreatitis, but not CF. We propose that early detection of CFTR dysfunction, combined with personalized medicine approaches directed at restoring CFTR function, can potentially slow or even prevent disease progression in some individuals affected by pancreatitis.

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Chapter 6

Pro-inflammatory cytokines stimulate CFTR-dependent anion secretion in pancreatic ductal epithelium

Dora Angyal[1], Tessa A. Groeneweg[1], Anny Leung[1], Max Desain[1], Kalyan Dulla[2], Hugo R. de Jonge[1]#, Marcel J.C. Bijvelds[1]
#Deceased.

[1]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, PO Box 2040, 3000CA Rotterdam, The Netherlands.
[2]Boehringer Ingelheim Pharma GmbH & Co. KG, Binger Strasse 173, 55216, Ingelheim am Rhein, Germany.

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Part II

Abstract

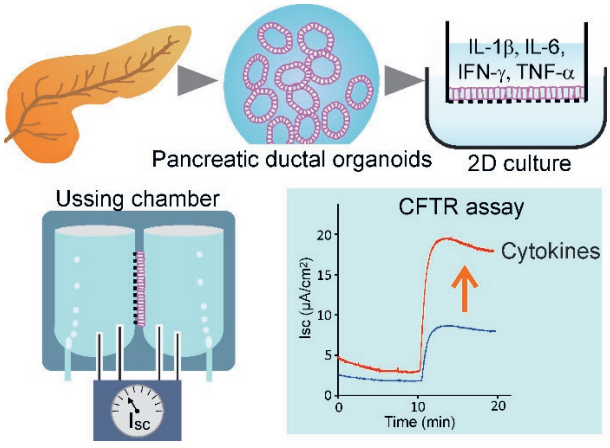
Background: Loss of CFTR-dependent anion and fluid secretion in the ducts of the exocrine pancreas is thought to contribute to the development of pancreatitis, but little is known about the impact of inflammation on ductal CFTR function. Here we used adult stem cell-derived cell cultures (organoids) obtained from porcine pancreas to evaluate the effects of pro-inflammatory cytokines on CFTR function.

Methods: Organoids were cultured from porcine pancreas and used to prepare ductal epithelial monolayers. Monolayers were characterized by immunocytochemistry. Epithelial bicarbonate and chloride secretion, and the effect of IL-1β, IL-6, IFN-γ, and TNF-α on CFTR function was assessed by electrophysiology.

Results: Immunolocalization of ductal markers, including CFTR, keratin 7, and zonula occludens 1, demonstrated that organoid-derived cells formed a highly polarized epithelium. Stimulation by secretin or VIP triggered CFTR-dependent anion secretion across epithelial monolayers, whereas purinergic receptor stimulation by UTP, elicited CFTR-independent anion secretion. Most of the anion secretory response was attributable to bicarbonate transport. The combination of IL-1β, IL-6, IFN-γ, and TNF-α markedly enhanced CFTR expression and anion secretion across ductal epithelial monolayers, whereas these cytokines had little effect when tested separately. Although TNF-α triggered apoptotic signaling, epithelial barrier function was not significantly affected by cytokine exposure.

Conclusions: Pro-inflammatory cytokines enhance CFTR-dependent anion secretion across pancreatic ductal epithelium. We propose that up-regulation of CFTR in the early stages of the inflammatory response, may serve to promote the removal of pathogenic stimuli from the ductal tree, and limit tissue injury.

Keywords: CFTR; Cystic Fibrosis; Cytokines; Epithelial ion transport; Organoid; Pancreatitis



Introduction

Acute pancreatitis is an inflammatory disease of the exocrine pancreas, which may develop under sterile conditions and is thought to be instigated by acinar cell injury (1, 2). Apart from transient obstruction of the pancreatic ducts by gallstones, which causes bile to enter the pancreatic ductal tree, the most common causes of acute pancreatitis are alcohol abuse and hypertriglyceridemia (2, 3). The inflammatory response may resolve without specific treatment within a few days, or progress to systemic inflammation, culminating in a life-threatening failure of organs outside the pancreas (2). Acute pancreatitis is relatively prevalent, accounting for a substantial portion of the hospitalizations that are related to the gastrointestinal tract (2, 4). Treatment relies principally on supportive care.

The digestive enzyme-producing acinar cells of the exocrine pancreas seem to be especially susceptible to pathogenic stimuli. Typical stressors associated with pancreatitis, like ethanol or bile acids are thought to disrupt Ca^{2+} homeostasis in these cells, leading to the untimely activation of proteolytic enzymes, principally trypsin, and cell death (1-4). Pathogenic stimuli also promote the activity of NFκB in acinar cells, which, together with the release of damage-associated molecular patterns (DAMPs) by injured cells, leads to the activation of stellate cells and immune cell infiltration (3-5). The release of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), by antigen presenting cells (dendritic cells, macrophages), and interferon γ (IFN-γ) by Th1 cells, causes further tissue damage, and may also promote the translocation of bacteria across the intestinal mucosa, leading to exposure of the pancreas to further pro-inflammatory stimuli (3-6).

Multiple genetic factors influence the susceptibility to environmental triggers of pancreatitis (7-9). One such factor is the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, which encodes a phosphorylation-regulated chloride/bicarbonate channel, principally expressed in epithelia of the respiratory and gastrointestinal tract. Loss-of-function mutations in *CFTR* cause cystic fibrosis (CF), a complex, multi-organ disease, with potentially fatal outcome (10). In the pancreatic ductal epithelium, CFTR-mediated anion transport is essential for the formation of an alkaline, bicarbonate-rich fluid (10, 11). In the majority of CF patients, i.e. those carrying *CFTR* alleles with little or no residual function, obstruction of the ducts by mucus and protein aggregates leads to atrophy and fibrosis, and exocrine pancreatic insufficiency develops early in life (10). Importantly, some more benign mutations in the CFTR gene, while not linked to early onset CF-typical pancreatic fibrosis and insufficiency, seem to predispose to the development of pancreatitis, and it has been estimated that circa 30% of individuals diagnosed with idiopathic pancreatitis carry mutations in *CFTR* (12-16). Pancreatitis is also a typical manifestation of CFTR-related disorders, a range of disorders not meeting criteria for CF diagnosis, but with evidence for moderate CFTR

dysfunction, and CF-like pathology often presenting in a single organ (17). Collectively, these findings suggest that the pancreas is eminently sensitive to impairment of CFTR function, and that even a subtle reduction in CFTR activity renders the pancreas more vulnerable to an environmental stressor or a mutation in another gene associated with pancreatitis, i.e. a second hit (1, 2, 7, 12, 15, 18).

Consistent with the concept that ductal CFTR has an essential tissue-protective role in the pancreas by containing the risk of tissue damage upon exposure to pathogenic stimuli, it was proposed that a reduction in ductal fluid secretion initiates and/or propagates some acute forms of pancreatitis. Typical stressors associated with pancreatitis, like trypsin, bile acids, ethanol and palmitoleic acid (which may be elevated in hypertriglyceridemia) were all shown to acutely reduce anion secretion in pancreatic ducts (18-21). However, little is known about the initial effects of inflammatory mediators released by infiltrating immune cells on CFTR activity in the pancreatic ductal epithelium. This question is pertinent, in view of the fact that several cytokines were shown to regulate the activity of CFTR and other anion transport mechanisms in epithelial tissues. For instance, it was reported that IL-1β and TNF-α stimulate CFTR-mediated fluid secretion in the submucosal glands of the respiratory tract, and that IL-1β induces expression of CFTR in human airway epithelium-derived Calu-3 cells (22, 23). Further, interleukin 4 (IL-4) was shown to stimulate CFTR-mediated chloride secretion across airway surface epithelia via a pendrin (*SLC26A4*)-dependent mechanism (24). Similarly, the combination of TNF-α and interleukin 17 (IL-17) was reported to strongly induce *SLC26A4* in airway epithelium, and to alkalinize airway surface liquid through stimulation of CFTR-dependent bicarbonate secretion (25). Cytokine-dependent alkalization of airway surface liquid, by stimulating mucociliary clearance and killing of bacteria, is thought to serve as a host-protective mechanism (26). Conversely, in intestinal and biliary epithelial cells, some of the same cytokines seem to repress CFTR activity, indicating that the effects of cytokines differ between tissues (27-29).

In the present study, we sought to further delineate the impact of inflammation on anion secretion across the pancreatic ductal epithelium. For this purpose, we cultured epithelial monolayers from porcine ductal organoids to quantitatively assess CFTR activity in pancreatic ductal epithelium. As in humans, the concentration of bicarbonate in the pancreatic juice produced by pigs is exceptionally high (>130 mmol/L), and porcine ductal anion and water secretion is highly dependent on CFTR (11, 30, 31). The latter is poignantly illustrated by the observation that CFTR deficient piglets display severe exocrine pancreatic destruction, starting from an early stage in life, i.e. a phenotype that closely resembles human CF pathology (32, 33). We found that monolayers derived from primary cultures of ductal cells form a highly polarized epithelium, that recapitulates many structural and functional aspects of the native

ductal epithelium. We used this model to characterize ductal anion transport and to assess the effect of cytokines typically associated with pancreatitis on CFTR function.

Materials and Methods

Animals

For culture of organoids, tissue was obtained from crossbred Yorkshire-Landrace pigs (*Sus scrofa*), aged 10-16 weeks. Pigs were anesthetized and euthanized as published previously (34). After excision, pancreatic tissue was briefly rinsed with and subsequently stored in ice-cold Ad-DF+++ medium: advanced DMEM/F12 (Gibco), HEPES (10 mmol/L, Gibco), Glutamax (2 mmol/L, Gibco) and Primocin (100 mg/L, InvivoGen). Studies were approved by the Ethical Committee for Animal Experiments of the Erasmus MC (AVD1010020173286).

Culture of pancreatic ductal organoids and epithelial monolayers

Tissue, collected from the head region of the pancreas, was minced into small fragments, circa 8 mm^3 in size, and tissue was further dissociated by treatment with collagenase, according to established protocols (35). After dissociation, ductal structures were selected using a bright-field microscope (Zeiss Primovert), and embedded in extracellular matrix (Matrigel, Corning). Tissue was incubated in Ad-DF+++ containing the growth factors Wnt3a, Noggin, R-Spondin 1, FGF10 and EGF, and PGE_2 and forskolin (35). To stimulate organoid development, Y-27632 (10 µmol/L; R&D Systems) was added during the first two days after seeding and passaging. Pancreatic ductal organoids could be expanded for up to 12 generations/passages.

For culture of epithelial monolayers, extracellular matrix-embedded, pancreatic organoids were enzymatically dissociated in TrypLE Express (Thermo Fisher). Because the rate of expansion of organoid lines generally decreased at later passages, we only used organoids at early passages (3-6) for preparing monolayers. After enzyme activity was quenched by addition of ice-cold Ad-DF+++, cells were filtered through a cell strainer (70 µm; Falcon), collected by centrifugation, and suspended in complete organoid culture medium, supplemented with Y-27632. Cells were seeded ($6x10^5/cm^2$) either in 96-well plates (#3596, Corning) or on permeable inserts (Transwell #3470; Corning) that had been pretreated with Matrigel diluted in phosphate buffered saline (PBS; 1:20; 0.2 mL/cm^2) for 2h at 37°C. Culture medium was as for extracellular matrix-embedded organoids, except that, to suppress CFTR activity, the adenylyl cyclase agonist forskolin was removed from the medium one day prior to ion transport measurements. Cells were cultured until a confluent monolayer was obtained (10-14 days). In some instances, prior to assessment of anion transport, confluent monolayers were exposed to TNF-α (50 ng/mL; Peprotech), IFN-γ (200 ng/mL, Peprotech), IL-1β (10 ng/mL; Peprotech) and/or IL-6 (100 ng/mL; Peprotech) for 20h.

Part II

Cell culture

The human colonic adenocarcinoma cell line HT29-CL19A (#T8212; Applied Biological Materials) was cultured in DMEM (Gibco), supplemented with fetal bovine serum (10%; Gibco), penicillin (100 U/mL; Gibco) and streptomycin (100 µg/mL; Gibco). Cell lysates were prepared in NaCl (150 mmol/L), Tris/HCl pH 7.6 (25 mmol/L), Triton X100 (1%), sodium deoxycholate (1%), sodium dodecyl sulfate (0.1%), supplemented with a protease inhibitor cocktail (Roche). Prior to Western blot analysis, cell lysates were stored at -80°C.

Gene expression

Organoids were collected in ice-cold PBS and extracellular matrix was removed by washing twice. RNA was extracted from organoids and organoid-derived epithelial monolayers using the NucleoSpin RNA kit (Macherey-Nagel), and synthesis of cDNA was performed using PrimeScript RT Enzyme Master Mix (Takara Bio Inc.). Quantitative reverse transcriptase PCR (primer sequences shown in Table 1) was performed in SYBR Green I Master mix (Thermo Fisher Scientific) using the StepOnePlus RT-PCR platform (Applied Biosystems, Thermo Fisher Scientific). Median values of assays performed in triplicate were used to determine gene expression levels relative to *YWHAZ*.

Table 1. Primer sequences used for RT-PCR

Gene	Accession number	Forward	Reverse
ANO1 (TMEM16A)	XM_021082670.1	CACCTTCATGGAGCACTGGA	GGATGGTCCTCCTCCTCCTC
ANO6 (TMEM16F)	XM_005655713.3	CGCCAGGCAGAACTTGAGTA	CACGTGAGTACACTGTGCCT
CFTR	NM_001104950.1	TCATGCCGGGCACCATTAAA	CGCTTTGATGACACTCCTGT ATCTA
SLC4A2 (AE2)	XM_021078861.1	CCGGTTGATGATGTCGAGGT	CAGCTGGTCCCTGTGAAGTC
SLC4A4 (NBCe1)	NM_001030533.1	TCTTTGCGACAGGTCGTGTA	CTTCCACGCTCTCAGTGGAC
SLC9A1 (NHE1)	NM_001007103.1	CCCCCAAGGACCAGTTCATC	AGGTCTACCAGAGGCCGAAT
SLC12A2 (NKCC1)	XM_003123899.5	GGATGGCAAGACTCCAACTCA	CCTCCATCATCAAAAAGCCA CC
SLC26A4 (Pendrin)	XM_003357511.4	TGGTGGGATCTGTTGTTCTGA	ACTCTTGCTGCGTCTCTAGC
SLC26A6 (PAT1)	NM_001012298.2	ATGGGGCTGTCAGAAGCG	GCCTCCTCAGCTCCATTGTT
YWHAZ	NM_001315726.1	GGAGCCCGTAGGTCATCTTG	ATTCTCGAGCCATCTGCTGT

For transcriptome analysis, library construction and sequencing was performed at the Beijing Genomics Institute (BGI). Data were processed with CLC genomic workbench 20 (Qiagen) and sequence reads were mapped to the *Sus scrofa* genome data set 11 (NCBI), using default parameters. Data depict reads per 1000 base pair transcript per million reads mapped (RPKM). Datasets are available through NCBI-GEO repository GSE233579.

Cytochemistry and immunocytochemistry

Pancreatic tissue or epithelial monolayers were fixed in paraformaldehyde (4%) and embedded in paraffin. Hematoxylin-eosin (HE) staining was performed on 5 µm sections, following established protocols. Immuno-detection of ductal proteins was performed using antibodies directed against keratin 7 (M7018, 1:100; Agilent Dako;), SOX9 (AMAb90795, 1:500; Atlas Antibodies), e-cadherin (#3195, 1:300; Cell Signaling Technology), zonula occludens 1 (21773-1-AP, 1:2000; Proteintech) or an affinity-purified polyclonal antibody directed against human CFTR (G449, 1:100), kindly provided by Dr. A.C. Nairn (Yale School of Medicine, New Haven) (36, 37). Sections were incubated with an appropriate *fluorescently*-tagged secondary antibody, i.e. goat-anti-mouse IgG (A-11032, Invitrogen) or goat-anti-rabbit IgG (A-11008, Invitrogen), and mounted in Vectashield mounting medium supplemented with 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei (Vector Laboratories). Fluorescence was visualized on a Stellaris 5 low incidence angle upright confocal microscope with a HC PL APO CS2 40x/1.30 oil objective (Leica Microsystems).

Western blot analysis

To assess cAMP-dependent protein kinase (PKA)-mediated phosphorylation of the vasodilator-stimulated phosphoprotein (VASP), organoids (9 days after seeding) were incubated for 30 min with vasoactive intestinal peptide (VIP; 50 nmol/L; Bachem), secretin (50 nmol/L; Bachem), or forskolin (10 µmol/L) in Meyler solution (mmol/L): 128 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose, at 37°C. Meyler solution was pre-equilibrated at pH 7.3, in a 5% CO_2 atmosphere. Subsequently, organoids were collected in ice-cold PBS supplemented with NaF (1 mmol/L). After centrifugation (300x g, 5 min), collected organoids were lysed in NaCl (150 mmol/L), Tris/HCl pH 7.6 (25 mmol/L), Triton X100 (1%), sodium deoxycholate (1%), sodium dodecyl sulfate (0.1%), NaF (5 mmol/L), Na_3VO_4 (3 mmol/L), supplemented with a protease inhibitor cocktail (Roche). Lysates were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membrane. VASP and Ser-157-phosphorylated VASP (pVASP) was detected using antibodies #3132 and #3111 (1:1000; Cell Signaling Technology), respectively. For detection of CFTR, a combination of 3 monoclonal antibodies was used (#432, #450, #596; 1:3000 each), kindly provided by Dr. J. Riordan (University of North Carolina, Chapel Hill) and the

Cystic Fibrosis Foundation, through the CFTR antibody distribution program (https://cftrantibodies.web.unc.edu/). Activated, cleaved caspase-3 was detected using antibody #9661 (1:1000, Cell Signaling Technology). Detection of β-actin (Sc-47778, 1:5000; Santa Cruz) or e-cadherin (#3195; 1:1500; Cell Signaling Technology) served as a loading control. Primary antibodies were detected using an appropriate fluorescently-tagged secondary antibody (goat-anti-mouse IgG or goat anti-rabbit IgG; Invitrogen), and fluorescence was detected on an Odyssey imaging system (Licor Biosciences).

Electrophysiological assessment of epithelial anion transport

Monolayers were mounted in P2302T/P2300-type Ussing chambers (Physiologic Instruments, San Diego), and bathed in either of two solutions. Firstly, combined chloride and bicarbonate transport was assessed in Meyler solution. Secondly, for assessing bicarbonate transport, NaCl and KCl were replaced by the isethionate salts, and $CaCl_2$ and $MgCl_2$ by the acetic acid salts of these cations. Solutions were maintained at 37°C, and adjusted to pH 7.3 by continuous gassing with 95% O_2, 5% CO_2. For some experiments, a chloride free buffer was prepared as above, except that the bicarbonate concentration was set at 100 mmol/L by isosmotic substitution of sodium-isethionate for $NaHCO_3$ (mmol/L: 48 Na-isethionate, 4.7 K-isethionate, 1.3 $Ca(CH_3COO)_2$, 1.0 $Mg(CH_3COO)_2$, 100 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose). The pH of this solution was adjusted to 8.0 by gassing with 95% O_2, 5% CO_2. The osmolality of buffer solutions was routinely checked using a cryoscopic osmometer, and ranged between 294 and 305 mOsm/kg (Osmomat 30, Gonotec GmbH). The transepithelial potential difference across epithelial monolayers was clamped at 0 mV with a VVC-MC8 module (Physiologic Instruments), and the resulting short-circuit current (Isc) was recorded using a PowerLab 8/35 AD-converter and associated software (LabChart 8; AD Instruments).

CFTR activity was stimulated by addition of either secretin (50 nmol/L; Bachem), VIP (50 nmol/L; Bachem), or forskolin (10 μmol/L). Isc responses (ΔIsc) shown represent the difference between the maximum Isc recorded after stimulation, and the Isc just prior to stimulation. PPQ-102 (20 μmol/L; Tocris) and bumetanide (50 μmol/L; Sigma) were used to inhibit CFTR or NKCC1 activity, respectively. UTP (50 μmol/L; Sigma-Aldrich) was added to the luminal bath to stimulate purinergic receptors that couple to Ca^{2+}-dependent chloride channels (CaCC). UTP responses were assessed in the presence of PPQ-102.

Caspase-3 and -7 activity

Confluent monolayers in 96-wells plates were exposed to TNF-α (50 ng/mL; Peprotech), IFN-γ (200 ng/mL, Peprotech), IL-1β (10 ng/mL; Peprotech) and/or IL-6 (100 ng/mL; Peprotech) for 20h. Experiments were performed in culture medium in a

5% CO_2 atmosphere at 37°C. CAS3/7 activity was assessed using a commercially available assay kit (SensoLyte; AS-71141, AnaSpec). Fluorescence was measured over a 30 min period using a CytoFluor 4000 microplate reader (λ_{ex} 485/20 and λ_{em} 530/25 nm; Applied Biosystems). Assays were performed in triplicate, and caspase 3/7 activity was determined from the initial reaction velocity, i.e. the increase in relative fluorescence units (RFU)/min, as per instruction of the kit manufacturer. Values presented depict caspase 3/7 activity relative to a paired solvent control.

Epithelial permeability

To assess the effect of cytokines on epithelial barrier function, fluorescein isothiocyanate (FITC)–dextran (average molecular weight 4 kDa; 100 μg/ml; Sigma) was added to the apical compartment of monolayers cultured on Transwell inserts, in the presence or absence of a combination of cytokines (50 ng/mL TNF-α, 200 ng/mL IFN-γ, 10 ng/mL IL-1β, 100 ng/mL IL-6). After a 20h incubation period, FITC-dextran accrued in the basolateral compartment was measured using a CytoFluor 4000 microplate reader (λ_{ex} 485/20 and λ_{em} 530/25 nm). For assessing transepithelial electrical resistance (TEER), epithelial monolayers were mounted in Ussing chambers, bathed in Meyler solution, and the transepithelial potential difference was clamped at 0 mV, as described above. The clamping voltage was briefly (2 s) increased from 0 to 5 mV (⍰V), and the resulting change in Isc (ΔIsc) was monitored to calculate TEER using Ohm's law (R = ΔV / ΔIsc).

Statistical analysis

Data are presented as means ± standard deviation. Differences between means were statistically analyzed by Student's t-test (two-sided) or ANOVA, using the Dunnett correction to control for multiple comparisons (Prism 9; Graphpad software).

Results

Organoids and epithelial monolayers cultured from porcine pancreas have characteristics typical of ductal epithelium

Isolated sections of ductal epithelium formed spheroidal structures when cultured in an extracellular matrix. Spheroids consisted of a uniform, single layer of columnar epithelial cells surrounding an apparently fluid-filled central lumen, in keeping with a ductal origin (Fig. 1A-D). Transcriptome analysis indicated that organoid cells express many genes which are characteristic of the pancreatic ductal epithelium, including structural proteins like keratin 7 and 8 (*KRT7*, *KRT8*), e-cadherin (*CDH1*), annexin A4 (*ANXA4*) and zonula occludens 1 (*TJP1*), and transcription factors (*SOX9, HNF1B*; Fig. 1E). Crucially, we also detected transcripts encoding proteins involved in ductal anion secretion. We observed robust expression of the secretin receptor (*SCTR*) as well as the purinergic P2Y2 receptor (*P2RY2*), but comparatively low transcript levels of the type 1 VIP receptor (*VIPR1*) and the somatostatin receptor (*SSTR1*). Cells expressed the principle cellular bicarbonate and chloride uptake mechanisms, i.e. the sodium-coupled cotransporters NBCe1 (*SLC4A4*) and NKCC1 (*SLC12A2*), respectively, the chloride-bicarbonate exchanger AE2 (*SLC4A2*), and the sodium-proton exchanger NHE1 (*SLC9A1*) which, through proton extrusion, contributes to intracellular alkalization (38). Further, we detected expression of *CFTR* and the chloride-bicarbonate exchanger PAT1 (*SLC26A6*), both key anion transport mechanisms involved in ductal bicarbonate secretion (38). Cells also expressed the WNK1 and SPAK (*STK39*) protein kinases and IRBIT (inositol 1,4,5-triphosphate (IP_3) receptors binding protein released with IP_3; *AHCYL1*), thought to be involved in the regulation of ductal bicarbonate secretion (39, 40). Interestingly, we observed high transcript levels of anoctamin 6/TMEM16F (*ANO6*), a protein that translocates phospholipids between the inner and outer leaflet of the plasma membrane, but, more tentative, may also function as a Ca^{2+}-dependent chloride channel (CaCC) (39, 41). In contrast, the expression of anoctamin 1/TMEM16A (*ANO1*), a bona fide epithelial CaCC, was comparatively low. Finally, transcript levels of genes typically expressed in pancreatic acinar or islet cells were generally low (42).

The presence of CFTR protein in ductal organoids was confirmed by Western blot analysis (Fig. 2, Supplementary data: Fig. S1). Using a combination of three monoclonal antibodies, a diffuse immunoreactive band was detected in cell lysates, distinctive of the mature, fully glycosylated protein (*band C*), with an estimated molecular mass of circa 160 kDa. For comparison, CFTR was also detected in HT29-CL19A cells, a human intestinal cell line frequently used to study CFTR function. In line with previous studies we found that the electrophoretic mobility of porcine CFTR is slightly higher than that of the human orthologue (33).

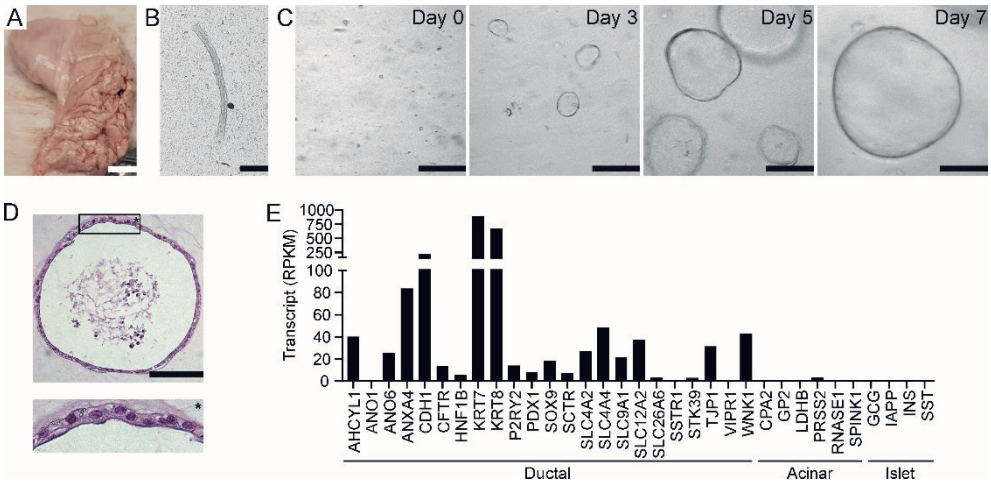


Figure 1. Establishment of pancreatic ductal organoids. (A) Excised pancreatic tissue. Scale bar: 1 cm. (B) Isolated duct (bright-field microscopy). Scale bar: 100 µm. (C) Development of spheroid organoids over the first seven days after seeding. (bright-field microscopy). Scale bars: 1 mm. (D) A pancreatic organoid (HE stain). Scale bar: 100 µm. (E) Transcript levels of ductal, acinar and islet cell markers.

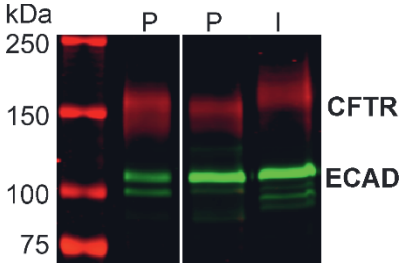


Figure 2. Detection of CFTR protein by Western blot analysis in two pancreatic organoid lines (P) and the human intestinal cell line HT29-CL19A (I). Detection of e-cadherin (ECAD) served as a loading control. Outer left lane shows the position of molecular weight markers, with an approximate molecular mass as indicated.

When cells derived from these ductal organoids were seeded on a permeable substrate they formed confluent monolayers within 1-2 weeks (Fig. 3A). We used immunocytochemistry to characterize these monolayers, and to compare the cellular partitioning of proteins to that of native pancreatic tissue. Cultured cells, as well as ductal epithelial cells in pancreatic tissue, showed nuclear localization of the transcription factor SOX9 (Fig. 3B), and stained positive for the ductal epithelial marker keratin 7 (KRT7; Fig. 3C). The cell-to-cell adhesion molecule e-cadherin (ECAD, *CDH1*) was localized to the lateral plasma membrane of cells in pancreatic tissue and monolayers, suggesting the formation of adherens junctions (Fig. 3D). Zonula occludens 1 (ZO-1; *TJP1*) was localized to the lumen-facing cell surface in ductal tissue, at the junction between adjacent cells, congruent with its function in the formation of tight junction complexes (Fig. 3E). A similar partitioning was observed in monolayers, indicating the formation of a highly polarized epithelium. Crucially, this was also suggested by the localization of CFTR, which was confined to the apical plasma membrane of cells in monolayers (Fig. 3F). In pancreatic tissue, CFTR was localized exclusively to the luminal membrane of the ducts.

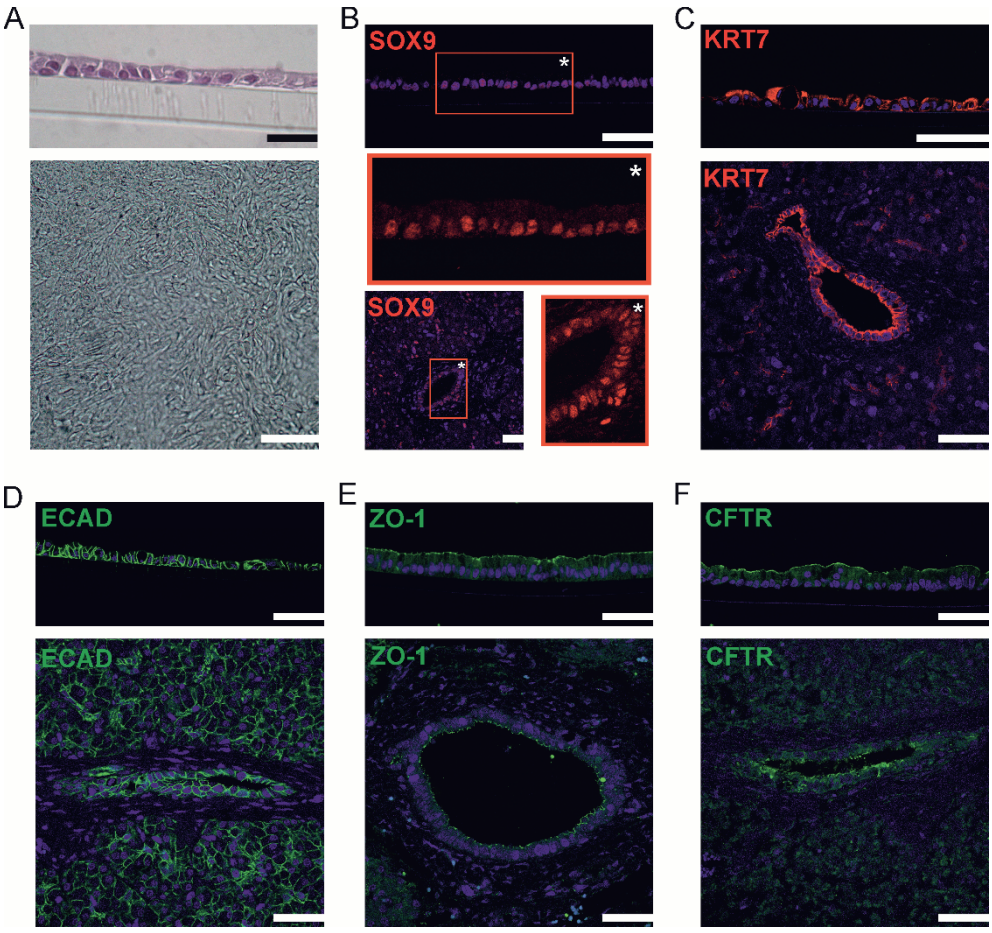


Figure 3. Pancreatic organoid-derived cells form a polarized epithelium when cultured on a permeable substrate. (A) Top panel: transverse section of an epithelial monolayer cultured on a Transwell insert (HE-stain). Scale bar: 25 µm. Bottom panel: the apical aspect of a confluent monolayer (bright-field microscopy). Scale bar: 100 µm. (B) Epithelial monolayers (top) and pancreatic tissue (bottom) were stained for SOX9. Nuclei are visualized using DAPI staining. Insets show SOX9 staining only in the area denoted by an asterisk. Scale bars: 50 µm. (C-F) Epithelial monolayers (top panels) and pancreatic tissue (bottom panels) were stained for KRT7, ECAD, ZO-1 or CFTR. Nuclei are visualized using DAPI staining. Scale bars: 50µm. Fluorescence was visualized on a Stellaris 5 low incidence angle upright confocal microscope with a HC PL APO CS2 40x/1.30 oil objective (Leica Microsystems).

Ductal epithelial monolayers display CFTR- and CaCC-mediated anion secretion

The intestinal hormone secretin and the neuronal signaling peptide VIP are known to control ductal fluid secretion through activation of Gs-coupled receptors that promote the production of cAMP and activation of PKA (39). To assess whether ductal organoids express functional secretin and/or VIP receptors, we analyzed phosphorylation of the PKA substrate VASP, an adaptor protein that is associated with the cell cytoskeleton (43). We observed that stimulation of organoids with either secretin, VIP, or the direct adenylyl cyclase agonist forskolin, triggered robust PKA-mediated Ser-157 phosphorylation of VASP (Fig. 4A, Supplementary data: Fig. S2, S3).

Next, we sought to ascertain whether activation of this (neuro)endocrine signaling pathway leads up to an anion secretory response. To this end, we assessed electrogenic ion transport across ductal epithelial monolayers. We observed that secretin, VIP and forskolin acutely triggered anion secretion (Fig. 4B). This response was blocked by the CFTR inhibitor PPQ-102. We consistently observed cAMP- and CFTR-dependent anion secretion in all cultures that were established from different animals. However, the magnitude of the response varied considerably between different preparations (Fig. 4C).

An important function of the ductal epithelium is the secretion of bicarbonate. Bicarbonate secretion may be mediated by CFTR directly, or by the chloride-bicarbonate exchanger PAT1 (*SLC26A6*), which activity may depend on the operation of CFTR as a chloride shunt (38, 39). To estimate the contribution of bicarbonate secretion to the forskolin-dependent Isc response, we used bumetanide to block NKCC1-dependent chloride secretion. We found that, in the presence of bumetanide, 73±5% (n=5) of the Isc response was retained (Fig. 4D). Next, we measured the forskolin-dependent Isc response in a bathing solution that contained bicarbonate (20 mmol/L), but no chloride or any other CFTR-permeating anion except bicarbonate. The absence of extracellular chloride excludes any possible contribution of exchangers like PAT1 to bicarbonate secretion. Consistent with the minor effect of bumetanide, we observed that the removal of chloride attenuated the forskolin-dependent Isc response, but that most of the Isc response was retained and must be attributed to bicarbonate secretion (65±4%; n=3; Fig. 4E). To mimic conditions in the distal part of the ductal tract, we also assessed bicarbonate secretion at a high luminal bicarbonate concentration (100 mmol/L), while keeping the bicarbonate concentration of the contra-luminal bathing solution at 20 mmol/L. We found that, even in the presence of such an unfavorable concentration gradient, the epithelial monolayers secreted bicarbonate when stimulated with forskolin (Fig. 4E, 4F).

Purinergic receptor activation, through increasing the cellular Ca^{2+} concentration, is known to activate CaCCs in various gastrointestinal epithelia, including that of the

pancreatic ductal tree (44). Presently, we observed that the purinergic receptor agonist UTP, when applied to the luminal bathing solution, elicited an anion secretory response in ductal epithelial monolayers (Fig. 4G). The effects of UTP were assessed in the presence of PPQ-102, indicating that these responses were mediated by an anion channel other than CFTR. The molecular identity of this CaCC is uncertain. A plausible candidate is anoctamin 1/TMEM16A, but based on our transcriptome data and previous reports of the anion channel properties of members of the anoctamin family, we cannot exclude that anoctamin 6/TMEM16F may also contribute to this activity (41, 44).

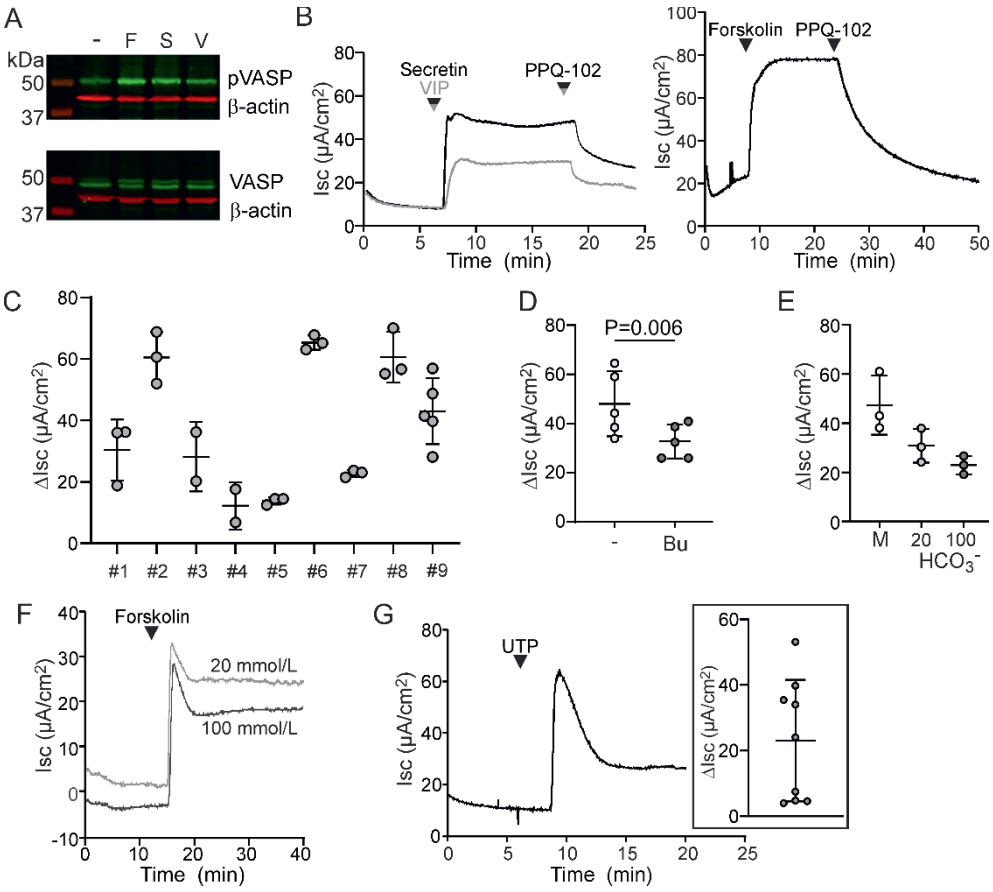


Figure 4. Pancreatic organoid-derived epithelial monolayers recapitulate ductal anion secretory pathways. (A) The effect of forskolin (F), secretin (S) and VIP (V) on PKA-mediated phosphorylation of VASP (top panel) and total VASP (bottom panel) levels was assessed by Western blot analysis. Detection of β-actin served as a loading control. Note that phosphorylation of Ser-157 decreases the electrophoretic mobility of VASP. Outer left lane shows the position of molecular weight markers, with an approximate molecular mass as indicated. (B) Representative experiments showing the effect of secretin, VIP, forskolin and PPQ-102 on the anion secretory current. (C) The cAMP-dependent Isc responses in nine organoid lines obtained from different animals. Each data point represents one technical replicate. (D) Forskolin-dependent Isc responses in the presence or absence of bumetanide (Bu). Each data point represents one technical replicate. Data were obtained from two organoid lines. Statistical analysis: paired t-test. (E) Forskolin-dependent Isc responses of monolayers bathed in Meyler solution (M), or in bathing solution containing only bicarbonate as the CFTR-permeating anion. Bicarbonate secretion was assayed at luminal bicarbonate concentrations of 20 or 100 mmol/L, as indicated. Each data point represents one organoid line. (F) Representative experiment demonstrating the forskolin-dependent Isc response at luminal bicarbonate concentrations of 20 or 100 mmol/L. (G) Effect of UTP on the anion secretory current. The inset shows the peak UTP-dependent Isc response, assessed in the presence of PPQ-102, in nine organoid lines.

Pro-inflammatory cytokines enhance CFTR expression and CFTR-dependent anion transport in ductal epithelial monolayers

Pancreatitis is associated with immune cell infiltration and the release of pro-inflammatory cytokines by antigen-presenting cells of the immune system, upon activation by DAMPs released by injured acinar cells (4). Typical cytokines produced in this process are IL-1β, IL-6, IFN-γ and TNF-α (3, 6). Ductal organoids expressed all four receptors of these cytokines (Fig. 5A). As TNF-α is known to trigger cell death under some conditions, we first ascertained whether cytokine exposure stimulates apoptotic activity, and/or affects the integrity of the epithelial barrier (45, 46). To this end, ductal epithelial monolayers were incubated with cytokines for 20h, and caspase 3/7 activity and the permeability of the epithelium to fluorescein isothiocyanate (FITC)-dextran was measured. We found that TNF-α alone, or in combination with IL-1β , IL-6, or IFN-γ led to a circa two-fold increase in caspase 3/7 activity (Fig. 5B). Stimulation of caspase 3 cleavage by this combination of cytokines was confirmed by Western blot analysis (Fig. 5C, Supplementary data: Fig. S4). Cytokine treatment did not enhance translocation of FITC-dextran across ductal epithelial monolayers, suggesting that barrier function was not acutely affected by this moderate increase in apoptotic activity (Fig. 5D). Still, we observed that the transepithelial electrical resistance (TEER), which principally reflects the permeability of the epithelium to ions, tended to be lower in cytokine-exposed monolayers (Fig. 5E).

To investigate putative effects of IL-1β, IL-6, IFN-γ and TNF-α on the anion secretory capacity of the ductal epithelium, we assessed the effect of cytokine treatment on the expression of the principle ion transport mechanisms involved in ductal bicarbonate and chloride secretion. Previous studies have shown that these same cytokines, either individually or in combination, and at similar concentrations as applied in our present assays, modulate *CFTR* expression and/or channel activity in intestinal, biliary, and respiratory epithelial cells (22, 23, 25, 27-29). It has also been shown that pancreatitis is associated with the release of these inflammatory mediators (3-6). Strikingly, we noted that the combination of these cytokines markedly enhanced expression of *CFTR* (Fig. 6A). In contrast, expression of the chloride-bicarbonate exchanger pendrin (*SLC26A4*), the CaCC anoctamin 1 (ANO1) and the sodium-proton exchanger subtype 1 (*NHE1*) were decreased by cytokine treatment.

In view of the marked induction of *CFTR* by cytokine treatment, we asked whether cytokine treatment may also enhance CFTR-mediated anion secretion across ductal epithelial monolayers. Indeed, treatment of monolayers with a combination of all four cytokines led to a significant increase in the forskolin-dependent Isc response (Fig. 6B,C). Correspondingly, administration of the CFTR blocker PPQ-102 led to a more pronounced decrease in the Isc in cytokine-treated epithelial monolayers than in untreated controls (Fig. 6C). We did not find a similarly robust increase in CFTR activity

when we tested the cytokines individually (Fig. 6D). Only IL-6 combined with either IL-1β or TNF-α led to a significant increase in the secretory response, albeit that these combinations failed to mimic the strong effect of the complete cytokine combination. Collectively, these results indicated that stimulation by IL-1β , IL-6, IFN-γ and TNF-α, by transcriptional regulation of the *CFTR* gene, increased CFTR-mediated anion secretion in pancreatic ductal epithelium.

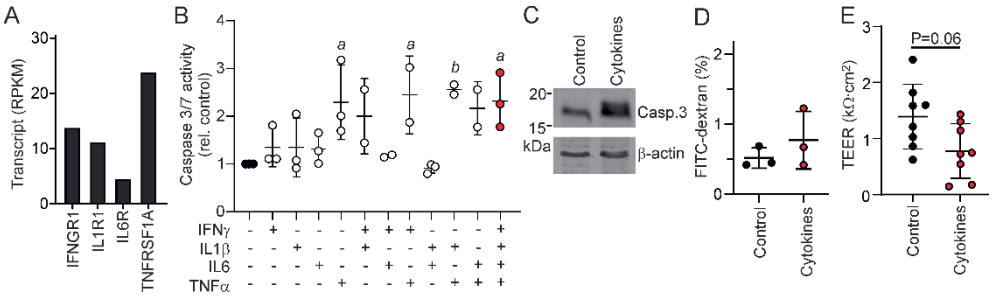


Figure 5. Effects of pro-inflammatory cytokines on apoptotic signaling and epithelial barrier function in pancreatic ductal organoids and epithelial monolayers. (A) Transcript levels of cytokine receptors. (B) Caspase 3/7 activity in epithelial monolayers after 20h exposure to cytokines. Each data point represents one technical replicate. Statistical analysis: ANOVA. [a] P=0.04, [b] P=0.02, compared to untreated. (C) Cleavage of caspase 3 in organoids upon incubation with IL-1β, IL-6, IFN-γ and TNF-α was assessed by Western blot analysis. Detection of β-actin served as a loading control. (D) FITC-dextran transport across epithelial monolayers in the presence or absence of IL-1β, IL-6, IFN-γ and TNF-α. Data represent the FITC-dextran levels in the contra-luminal compartment, relative to the concentration in the luminal compartment at the start of the experiment. Each data point represents one technical replicate. (E) Transepithelial electrical resistance (TEER) of epithelial monolayers mounted in Ussing chambers, in the presence or absence of IL-1β, IL-6, IFN-γ and TNF-α. Each data point represents one technical replicate. Statistical analysis: t-test.

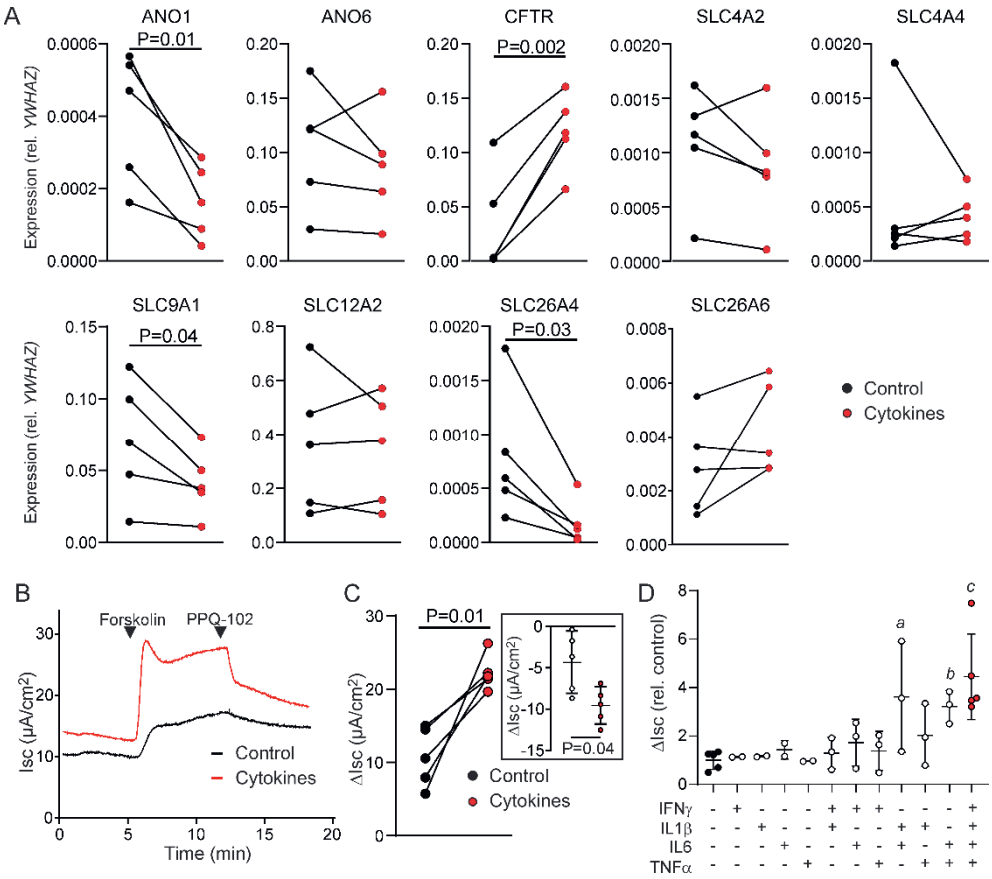


Figure 6. Effect of pro-inflammatory cytokines on anion transport in pancreatic ductal organoid-derived epithelial monolayers. (A) Effect of combined IL-1β, IL-6, IFN-γ and TNF-α exposure (20h) on the expression of genes involved in ductal anion secretion. (B) Representative experiment showing the effect of combined IL-1β, IL-6, IFN-γ and TNF-α exposure (20h) on the forskolin-dependent, PPQ-102-sensitive, Isc response. (C) Forskolin-dependent Isc responses in cytokine-treated and control monolayers. The inset shows the change in Isc elicited by PPQ-102. (D) The effect of different cytokines and combinations thereof on the CFTR-mediated Isc response. Experiments were performed on cultures obtained from three animals. Connecting lines indicate paired experiments performed in parallel, on monolayers cultured from the same organoid line. Statistical analysis: paired t-test (A,C), ANOVA (D). [a] P=0.02, [b] P=0.007, [c] P<0.001, compared to untreated.

Discussion

In this study, we characterized pancreatic ductal anion (bicarbonate, chloride) transport and assessed the effect of cytokines typically associated with pancreatitis on ductal CFTR function. The pancreatic ductal epithelial model used in our study was based on the generation of adult stem cell-derived organoids, cultured from ducts of the porcine pancreas (35). We show that these organoids expressed typical ductal cell markers, including key transport mechanisms involved in transepithelial bicarbonate secretion, such as CFTR, PAT1 (*SLC26A6*), and NBCe1 (*SLC4A4*). In line with earlier work on porcine, bovine and human primary pancreatic ductal cell cultures, we demonstrate that cells derived from these organoids, when seeded on a permeable substrate, formed a highly polarized epithelium (47-50). When stimulated by either cAMP- (VIP, secretin) or Ca^{2+} (ATP/UTP)-linked neuro-/endo-/paracrine factors, ductal epithelial monolayers displayed anion secretion, congruent with previous studies on isolated native pancreatic ducts (51-53). Postprandial release of secretin by enteroendocrine cells and VIP by parasympathetic neurons strongly stimulates the bicarbonate and fluid output of the exocrine pancreas (30, 39). Indeed, we found that only circa a quarter of the current elicited by cAMP/PKA agonists was bumetanide-sensitive (i.e. dependent on NKCC1-mediated chloride uptake), strongly suggesting that most of the response represents bicarbonate secretion.

Although PAT1, which is thought to operate with a 1:2 Cl^-/HCO_3^- stoichiometry, may mediate net anion secretion, we conclude that the current elicited by cAMP agonists in the presently studied epithelial monolayers is mediated predominantly or entirely by CFTR (39, 54). Firstly, because it was sensitive to pharmacological CFTR inhibition. Secondly, because we observed that in the absence of extracellular chloride, which, because their operation is fully dependent on luminal chloride, excludes any involvement of SLC26-type anion exchangers, the monolayers still secreted bicarbonate. Previous work has shown that bicarbonate and fluid secretion across the pancreatic ductal epithelium is highly dependent on CFTR, but the exact role of CFTR in ductal bicarbonate secretion is not fully understood (11, 39). Ductal cells are thought to secrete bicarbonate principally via Cl^-/HCO_3^- exchangers belonging to the SLC26 family, like PAT1 (38, 39). In this scenario, CFTR may primarily be required to transport chloride, entering the cells via the exchanger, back to the lumen. However, as luminal chloride is replaced by bicarbonate, the driving force for Cl^-/HCO_3^- exchange is gradually diminished, and computational modeling predicts that SLC26-type exchangers contribute progressively less to cellular bicarbonate extrusion as the luminal concentration approaches the levels found in the final product (54). These considerations have led to the assumption that, to reach the final high bicarbonate concentrations observed in human and porcine pancreatic juice, bicarbonate extrusion from epithelial cells, at least in the distal section of the ducts, must principally be

channel (i.e. CFTR)-mediated (39, 54). Consistent with this view, our data demonstrate that bicarbonate secretion across ductal epithelium can occur directly via CFTR, without involvement of PAT1 or any other apically located Cl^-/HCO_3^- exchanger. Moreover, we observed robust cAMP-dependent bicarbonate secretion in monolayers that were exposed to luminal bicarbonate concentrations approaching those encountered in the distal section of the ductal tree, demonstrating that ductal epithelial cells can sustain a driving force for bicarbonate efflux, even in the presence of a steep, opposing concentration gradient.

Even though all organoid lines were cultured from ducts isolated from an anatomically defined region of the pancreas, we observed marked differences in the amplitude of the cAMP-dependent anion secretory response between preparations. Each culture was initiated from only a limited amount of ductal fragments, and it is conceivable that separate organoid lines were derived from stem cells originating from different parts of the ductal tree. Immunolocalization and in situ hybridization studies indicate that CFTR can be found in all sections of the pancreatic ductal epithelium, but that its levels vary, and that expression is most prominent in the intralobular and centroacinar cells of the proximal tract (55, 56). Therefore, we surmise that the variation observed in the secretory response of monolayers, may reflect regional differences in the expression and activity of CFTR in the ductal epithelium. In addition, regional variation in the expression and activity of other key transporters (e.g. NBCe1, NKCC1) may also affect anion secretory capacity. Further, we cannot exclude that other factors, such as genetic variation between individual animals, play a role. In all organoid lines that were established, we also detected UTP-dependent anion secretory responses, consistent with the expression of purinergic P2Y2 receptors. As for CFTR, there is evidence for the presence of CaCC activity in proximal as well as in distal sections of the ductal tree. ATP/UTP-dependent secretory responses have previously been detected in cells isolated from the main (distal) ducts of the bovine pancreas, in mice intra-/interlobular ducts, guinea pig interlobular ducts, and in human pancreatic cell lines (44, 49, 52, 53, 57). The molecular identity of this CaCC has not been definitely established yet, but ANO1 (TMEM16A) appears to be the most plausible candidate (35, 44, 58).

Pancreatitis is associated with immune cell infiltration and the local, immediate release of pro-inflammatory cytokines (4). Our results indicate that select cytokines that are key mediators of the pancreatic inflammatory response markedly increased expression of CFTR in ductal epithelium, and stimulated CFTR-mediated ductal anion secretion. Induction of *CFTR* required a combination of pro-inflammatory cytokines, as individual cytokines were ineffective. Similar has been observed for epithelia of the biliary and respiratory tract (23-25, 29). However, although enhancing *CFTR* expression, the combination of IL-1β, IL-6 and IFN-γ lowered cAMP-dependent anion secretion across bile duct epithelium, indicating cytokines affect the expression or activity of additional

ion transport mechanisms or enzymes involved in biliary ductal anion secretion (29). Stimulatory effects of cytokines on CFTR activity have previously been described for epithelia of the airways, but, to our knowledge, the effect of immune modulators on CFTR function in the pancreatic ductal epithelium has not been investigated. For lung tissue and cell models, it has been shown that a combination of cytokines involved in airway host defense promote CFTR expression and enhance CFTR-mediated bicarbonate secretion (22-24). By increasing luminal pH, this is thought to improve mucociliary clearance and killing of bacteria, and, consequently, play an important role in the host defense response (25). We propose that up-regulation of CFTR in the pancreatic ducts by inflammatory mediators may, similarly, serve to accelerate the removal of pathogenic stimuli from the ductal tree, whether endogenous (bile acids, digestive enzymes) or environmental (microbial, chemical) stressors. In this manner, stimulation of CFTR activity could help to contain the progression of pancreatitis at an early stage, and limit tissue injury.

Our data indicate that the pancreas employs a different repertoire of cytokines to promote CFTR activity than the airways (22, 25). In both organs, the prototypical pro-inflammatory cytokines IL-1β and TNF-α appear to be involved, but the induction of *CFTR* in pancreatic epithelia did not require IL-17, whereas this cytokine was crucial in the response of airway epithelial cells (25). Conversely, it is unknown whether IL-6, which was essential for inducing *CFTR* in pancreatic ductal cells, affects *CFTR* expression and/or activity in respiratory epithelium. This disparity is probably reflective of the differences between both organs with respect to the specific cytokines involved in their innate and adaptive immunological defense mechanisms (4, 25, 59). Also, other than in airway epithelium, we found that cytokines did not increase the expression of the chloride-bicarbonate exchangers that potentially contribute to ductal bicarbonate secretion. Whereas expression of the Cl^-/HCO_3^- exchanger pendrin (*SLC26A4*) is strongly induced by pro-inflammatory cytokines in respiratory epithelium, in pancreatic cells, expression of *SLC26A4* was suppressed by cytokines and no induction of the principle Cl^-/HCO_3^- exchanger expressed in pancreatic ductal epithelium, PAT1 (*SLC26A6*), was observed (24, 25).

No *in vitro* model can fully emulate the complex immunological environment of pancreatitis *in vivo*. Consequently, our study has limitations. Firstly, only a select number of cytokines were probed, and we consider it probable that further signaling molecules secreted by immune- or epithelial cells play a role in the acute regulation of CFTR activity and ductal anion secretion. Secondly, we did not take into account the role of direct interactions of ductal epithelial cells with infiltrating immune cells or the involvement of stellate cells, which may have important immune modulatory roles (4, 60). Chronic inflammation of the pancreas is known to trigger fibrosis and lead to the gradual loss of ductal function, so it is also probable that the effects of prolonged

cytokine exposure are different from the more short-term effects investigated here (4, 7). For instance, we show that TNF-α only moderately stimulated pro-apoptotic signaling, but, upon prolonged exposure, this cytokine has been shown to impair epithelial barrier function (45, 46). Finally, epithelial monolayers derived from adult stem cell-based organoids may not be fully representative of the ductal epithelium *in vivo*, even though our experiments indicate that the epithelium formed by these cultured cells shares crucial structural and functional features with native ductal epithelium.

Conclusions

In conclusion, we show that CFTR activity in pancreatic ductal epithelium is enhanced by exposure to a combination of pro-inflammatory cytokines. Induction of *CFTR* in the initial stages of the inflammatory response, by enhancing the secretory capacity of the ductal epithelium, may serve to alleviate the effects of external stressors, attenuating or preventing further tissue injury. Our data are consistent with studies that point towards a more central role of CFTR dysfunction in the pathophysiology of pancreatitis, which indicate that, for some forms, anion secretory defects in the ductal compartment precede acinar pathology (18, 19, 21, 61). This concept may inform the development of new treatment modalities for pancreatitis. The discovery of small-molecule therapeutics that restore mutant CFTR function, has transformed the treatment of CF, leading to often marked improvements in clinical outcome. These so-called CFTR modulator drugs improved biomarkers of exocrine pancreas function and may reduce the risk of pancreatitis in CF patients (62, 63). In addition, modulators were shown to rescue pancreatic function in a non-CF model of pancreatitis, indicating that they may also improve wild type CFTR function (18). Taken together, these findings suggest that drug therapies directed at improving CFTR function, or ductal anion and fluid secretion in general, may be applicable for management of pancreatitis in a wider population.

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Chapter 7

Ethanol and unsaturated fatty acids do not impair CFTR function in primary human pancreatic ductal cells

Dora Angyal, Layla Moesker, Marco J. Bruno, Maikel P. Peppelenbosch, Marcel J.C. Bijvelds

Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

In preparation

Alcohol abuse is a common cause of acute pancreatitis that typically develops after a prolonged period of regular, immoderate ethanol consumption (>5 years of daily drinking). The etiology of alcohol-related pancreatitis is likely multifactorial, as several pathophysiological processes are thought to contribute to the development of pancreatic cell injury and inflammation. Primarily, ethanol has been demonstrated to perturb Ca^{2+} homeostasis in acinar cells, leading to mitochondrial dysfunction, the premature activation of trypsin, and release of inflammation mediators, including damage associated molecular patterns (DAMPs), which trigger immune cell infiltration (1-3). Apart from these direct effects on acinar cells, ethanol consumption increases circulating levels of potentially cytotoxic metabolites, including fatty acids and fatty acid ethyl esters (the latter are produced during nonoxidative ethanol metabolism) (4). At the onset of pancreatis, these products are thought to be released from intra-abdominal fat deposits, and to propagate the inflammatory response, by causing further acinar cell injury (4-6). In particular, unsaturated fatty acids (UFAs), including linoleic-, palmitoleic- (POA), and oleic acid (OA), more so than their ethyl ester derivatives, were shown to be toxic to acinar cells (4, 7-9).

Apart from their deleterious effects on acinar cells, ethanol and UFAs may also impair the function of the ductal compartment. Ductal secretion of fluid and base equivalents is critically dependent on CFTR, a chloride and bicarbonate channel that is principally expressed in epithelia lining the respiratory and gastrointestinal tract. The crucial role of CFTR in pancreatic physiology is exemplified by the fact that carriers of mutations in the *CFTR* gene are at a higher risk of developing pancreatitis, whereas those carrying loss-of-function mutations on both alleles develop cystic fibrosis (CF), a complex disease, associated with ductal atrophy, fibrosis, and exocrine pancreatic insufficiency (10, 11). The loss of CFTR-dependent ductal bicarbonate and fluid secretion is thought to impede the timely expulsion (flushing) of digestive enzymes from the ductal tract, leading to the accumulation of zymogen material (12). Importantly, both ethanol and POA were shown to promptly (within 30 min after application) impair fluid secretion in isolated pancreatic ducts of guinea pigs, suggestive of CFTR dysfunction (13). Consistent with this notion, whole cell patch clamp experiments performed on ductal cells from guinea pigs indicated that both ethanol and POA inhibit a current with CFTR-like properties (13, 14). The mechanism by which ethanol and POA exerted this acute inhibitory effect on ductal fluid secretion and CFTR function has not been completely resolved, but may involve mitochondrial dysfunction (13, 14). In addition, it was observed that long-term exposure (>6h) reduced CFTR localization at the cell surface, as well as its total abundance in ductal epithelial cells (13).

In view of their cytotoxic effects in acini, and their effect on anion and fluid transport in isolated guinea pig ducts and cells, we asked whether ethanol and/or UFAs affect CFTR function in human pancreatic ductal cells. To address this question, we cultured

epithelial monolayers from human pancreas-derived primary cultures of ductal cells (organoids), and assessed the effect of ethanol, OA, and POA on cell viability and CFTR-mediated anion secretion.

Firstly, to explore potential cytotoxic effects of UFAs and ethanol, we assessed phosphorylation/activation of the p38 mitogen-activated protein kinase (MAPK), a protein kinase that is activated by a variety of environmental stresses, to mitigate cell injury and promote survival (15). Ductal organoid-derived cells were exposed to concentrations of UFAs that are within the range encountered in inflamed pancreatic tissue, and an ethanol concentration in the upper range of those observed in subjects with acute alcoholic intoxication (4, 16). At these levels, OA, POA and ethanol were previously shown to promote substantial cell death in murine acini, *in vitro* (4, 9). We observed that incubation with OA or POA stimulated phosphorylation of p38 MAPK in ductal cells (Fig. 1A). Ethanol, in the absence of UFAs, did not stimulate p38 MAPK phosphorylation, and, when co-administered, did not enhance the effect of OA or POA.

To investigate the effect of ethanol and UFAs on cell viability, we assessed lactate dehydrogenase (LDH) release by ductal cells. Our data demonstrated that treatment of ductal cells with POA alone, or treatment with the combination of POA and ethanol, increased LDH release, compared to untreated controls (Fig. 1B). Still, the LDH release after POA exposure was only 1.1±1.5% (n=17) of the maximum release caused by cell lysis, indicating POA only modestly affected cell viability. OA also tended to increase LDH release, but the apparent difference with the control group failed to reach statistical significance. Ethanol, in the absence of UFAs, had no effect on LDH release.

Cells stemming from human pancreatic ductal organoids were then cultured on a permeable substrate to form confluent, epithelial monolayers. CFTR-dependent anion transport across these ductal epithelia was assessed by short circuit current (Isc) measurement. The cAMP agonist forskolin induced a sustained increase in the Isc, consistent with CFTR-mediated anion secretion. After the forskolin-dependent Isc response had reached a plateau, OA, POA, or POA combined with ethanol, were added to the bathing media, and the Isc was continuously monitored over 1h. We observed that, over this period, none of these treatments significantly attenuated the Isc response elicited by forskolin (Fig. 1C,D). However, in all instances, subsequent addition of the CFTR inhibitor BPO-27 promptly lowered the Isc (Fig. 1C,E). In these same experiments, we measured the transepithelial electrical resistance (TEER) before and after >1h UFA and/or ethanol exposure. We found that none of the tested compounds significantly reduced the TEER over this period, indicating that the permeability of the epithelium to ions was not affected by the exposure to UFAs or ethanol (Fig. 1F).

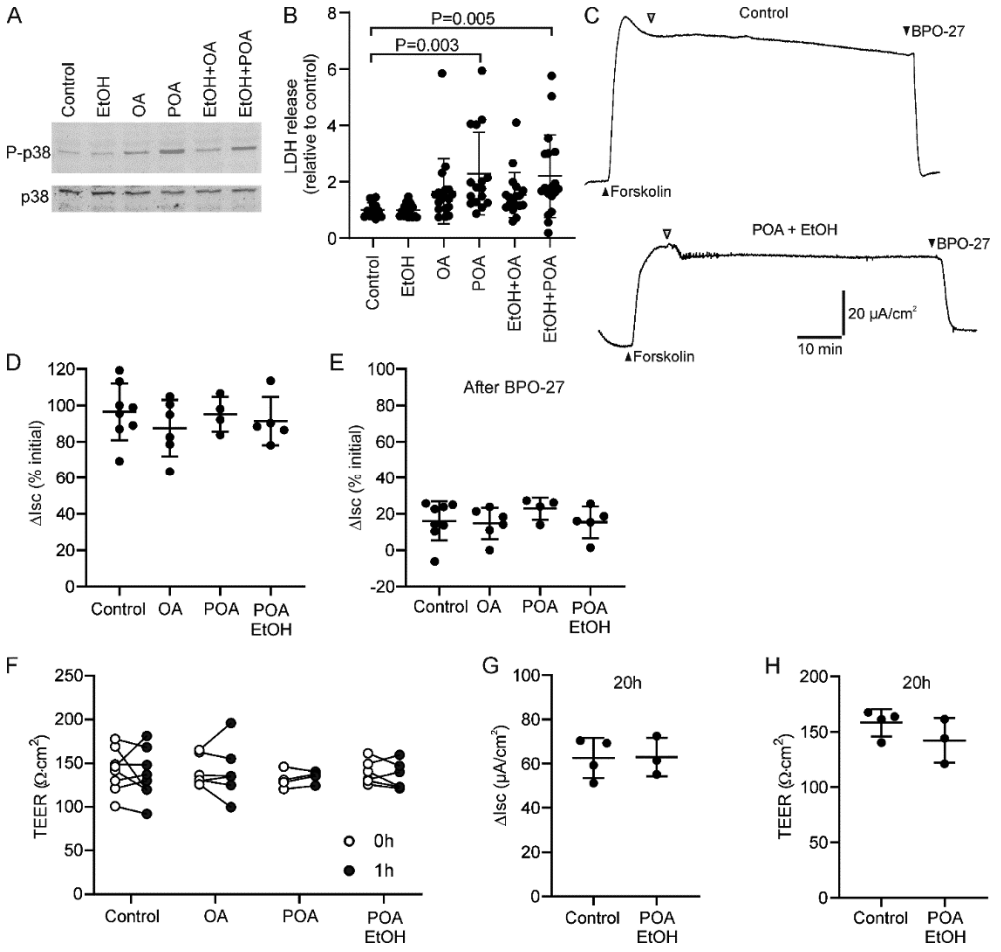


Figure 1. Effect of unsaturated fatty acids and ethanol on pancreatic ductal epithelium. (A) The level of phosphorylated p38 MAPK (P-p38) and total p38 MAPK was assessed by Western blot analysis. Lysates were prepared from ductal epithelial monolayers exposed for 30 min to OA (300 µmol/L), POA (200 µmol/L), and/or ethanol (EtOH; 100 mmol/L). (B) LDH release from ductal epithelial monolayers after exposure (4h) to OA (300 µmol/L), POA (200 µmol/L), and/or ethanol (100 mmol/L). (C) Representative recordings of the forskolin-dependent Isc response of epithelial monolayers. After vehicle (4 mmol/L ethanol; upper recording) or the combination of POA (200 µmol/L) and ethanol (100 mmol/L; bottom) were added (marked by shaded arrow), the Isc was monitored for 1h, before BPO-27 (10 µmol/L) was added. (D) Aggregate data of the effect of OA, POA and the combination of POA and ethanol on the forskolin-dependent Isc. Data shown represent the forskolin-dependent Isc, at 1h after the start of the fatty acid/ethanol exposure, relative to the response just before addition of the test compounds. (E) The forskolin-dependent Isc response in the presence of BPO-27, relative to the response recorded just before addition of the test compounds. (F) Transepithelial electrical resistance (TEER) of epithelial monolayers recorded at the start of the experiment (0h), and after exposure to OA, POA and/or ethanol (1h). – *Legend continued on next page.*

(G) Peak forskolin-dependent Isc responses of epithelial monolayers exposed for 20h to a combination of POA (200 µmol/L) and ethanol (100 mmol/L), and paired controls. (H). TEER of epithelial monolayers after 20h exposure to POA and ethanol. Horizontal bars in graphs depict mean ± SD. Each data point represents one technical replicate. Statistical analysis: ANOVA (B,D); paired Student *t* test (F); Student *t* test (G,H).

Because prolonged exposure to ethanol or POA was shown to reduce cellular CFTR abundance, we also assessed the forskolin-dependent Isc response of ductal, epithelial monolayers that were exposed to a combination of POA and ethanol for 20h (13). We observed that the forskolin-dependent Isc response of such monolayers was similar in magnitude to that of paired controls (Fig. 1G). Further, we noted that, likewise, the TEER of monolayers exposed for 20h to a combination of ethanol and POA was similar to the TEER of paired controls (Fig. 1H).

Finally, we asked whether POA and/or OA affected CFTR localization in epithelial monolayers. We exposed epithelial monolayers to either OA or POA for 1 h. In monolayers exposed to POA, CFTR localization at the plasma membrane was similar to that of untreated monolayers (Figure 2). Monolayers exposed to OA also showed apical CFTR localization. Examination at a lower laser intensity suggested that CFTR abundance at the apical aspect of the cells was lowered by OA treatment, compared to the control. This suggested that OA may affect CFTR localization.

In this study we demonstrated that neither ethanol, nor the UFAs OA and POA, impaired CFTR-mediated anion secretion across human pancreatic ductal epithelium, *in vitro*. Further, we found that the TEER of epithelial monolayers was unaffected by (prolonged) exposure to ethanol and UFAs, indicating that these compounds do not impact the epithelial barrier. Congruently, although OA or POA exposure prompted a cellular stress response, i.e. activation of the p38 MAPK, the immediate effects of UFAs on ductal cell viability were modest, in particular when compared to the previously reported prominent effects of UFAs on the viability of murine acinar cells (4, 5, 9). These data indicate that alcohol abuse is unlikely to impair CFTR-dependent ductal anion secretion, unless through secondary effects that result from acinar cell dysfunction and inflammation.

Previously, it was shown that POA and ethanol strongly inhibited cAMP-dependent fluid secretion in isolated pancreatic ducts of the guinea pig (13). The same study reported that POA and ethanol prevented the recovery of ductal cells from cellular alkalization, indicative of impaired bicarbonate efflux. Because, in whole cell patch clamp experiments, POA and ethanol inhibited a current with CFTR-like properties, it was inferred that the observed effects on ductal fluid secretion and cellular pH regulation were (partly) attributable to loss of CFTR activity. Evidently, these results differ from

our present data. Possibly, these differences are attributable to the different experimental models used. For instance, the guinea pig ducts and cells used in previous studies may be inherently more sensitive to UFA and ethanol exposure than the human ductal cells used presently. Further, it is conceivable that ethanol and POA target yet other anion transport mechanisms in pancreatic ducts, that were not evaluated in our present experiments, which focused on CFTR function. In this context, it is of interest that palmitoleic acid ethyl ester (POAEE), which is produced from POA during nonoxidative ethanol metabolism, failed to mimic the effects of POA and ethanol on ductal fluid secretion and cellular pH recovery in the guinea pig model. Still, in whole cell patch-clamp experiments on guinea pig ductal cells, administration of POAEE was shown to inhibit an apparently CFTR-mediated current (putatively, because POAEE is converted to POA by fatty acid ethyl ester hydrolases in ductal cells) (14). From these disparate effects of POAEE on ductal fluid transport *vs*. ductal CFTR activity, it may be inferred that the POA/ethanol-sensitive, but POAEE-insensitive, fluid- and bicarbonate secretory response observed in pancreatic ducts and ductal cells, respectively, involves (an) anion transport mechanism(s) different from CFTR. A potential candidate is the chloride/bicarbonate exchanger SLC26A6, which, like CFTR, mediates ductal bicarbonate secretion (17). This conjecture is consistent with the observation that combined ethanol/fatty acid administration impaired secretin-dependent pancreatic fluid secretion in CFTR-deficient mice, *in vivo*, which implies that the anti-secretory action of this combination is, at least in part, independent of the presence of CFTR (13).

In summary, the marginal effects of UFAs on ductal cell viability, and the absence of an effect on CFTR-mediated anion secretion observed presently, argue against a direct involvement of acquired CFTR dysfunction in the development of alcohol-related pancreatitis. While we cannot exclude the possibility that they may have more pronounced effects on the ductal epithelium when tested at higher concentrations, OA, POA, and ethanol, at the concentrations used in our present study, were previously shown to cause significant cytotoxicity in acinar cells (4, 9). This suggests that acinar cells are more susceptible to the noxious effects of UFAs and ethanol than the ductal epithelium, and, consequently, are the focus of alcohol-induced pancreatitis.

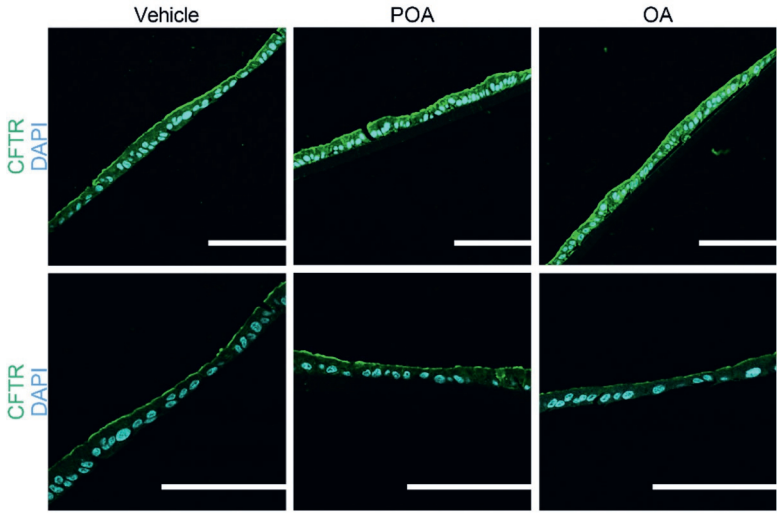


Figure 2. Human pancreatic ductal organoid-derived epithelial monolayers maintain apical CFTR expression upon fatty acid exposure. Immunocytochemistry was used to assess the effect of POA (200 µmol/L) and OA (300 µmol/L) on CFTR localization. Epithelial monolayers were stained for CFTR. Nuclei are visualized using DAPI. Fluorescence was visualized at high (top) or low (bottom) laser intensity (on a Stellaris 5 low incidence angle upright confocal microscope with a HC PL APO CS2 40x/1.30 oil objective (Leica Microsystems). Scale bars: 100 µm.

Part II

Methods

Organoid culture

Human pancreatic ductal organoids (hPDOs) from normal tissue were obtained from HUB Organoids, Utrecht, the Netherlands (MTA 21/034 and MTA 25/054). Organoids were maintained according to established protocols described elsewhere (18) in medium containing forskolin (10µmol/L, Bio-Connect BV) and prostaglandin E2 (PGE2) (1µmol/L, Bio-Techne), which were removed 5 days prior to experiments to suppress CFTR activity.

For culture of epithelial monolayers, organoids were enzymatically dissociated in TrypLE Express (Thermo Fisher). After enzyme activity was quenched by addition of ice-cold medium, cells were collected by centrifugation, resuspended, and seeded (6 × $10^5/cm^2$) on permeable inserts (Transwell #3470; Corning) or (1.5 x $10^5/cm^2$) in tissue-culture treated cell culture plates (Sarstedt) that had been pretreated with Matrigel diluted in phosphate buffered saline (PBS; 1:20; 0.2 mL/cm^2) for 2 h at 37 °C. Culture medium was as for extracellular matrix-embedded organoids. Cells were cultured until a confluent monolayer was obtained (10–14 days for Transwells, or 7-10 days for cell culture plates).

Preparation of unsaturated fatty acid stock solutions

Solutions (1 mol/L) of palmitoleic acid (POA, Sigma-Aldrich, Saint-Louis, USA) and oleic acid (OA, Sigma-Aldrich) were prepared in ethanol. Immediately before an experiment was conducted, these stocks were diluted (1:100) in HEPES-buffered saline (pH 7.3), and unsaturated fatty acids were dispersed by brief sonification. The resulting aqueous solution was further diluted in Meyler solution (mmol/L: 128 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose), or, in case >4h incubation was required, organoid culture medium.

Electrophysiological assessment of epithelial anion transport

Epithelial monolayers cultured on permeable inserts were mounted in P2302T/P2300-type Ussing chambers (Physiologic Instruments, San Diego). Monolayers were bathed in Meyler solution maintained at 37°C, gassed with 95% O_2, 5% CO_2. The transepithelial potential difference was clamped at 0 mV with a VVC-MC8 module (Physiologic Instruments) and the resulting short-circuit current (Isc) was recorded using a PowerLab 8/35 recording unit and associated software (LabChart 8; AD Instruments). The transepithelial electrical resistance (TEER) of epithelial monolayers was assessed applying Ohm's law, by monitoring the Isc response that resulted from briefly (2 s) clamping the voltage at -5 mV.

Cytotoxicity assay

Organoid-derived ductal epithelial cells cultured in 96-well cell culture plates were incubated with POA, OA, and/or ethanol in Meyler solution (37°C; 5% CO_2). After 4h, the extracellular medium was sampled, and LDH activity was analyzed using a reagent kit (CyQUANT™ LDH Cytotoxicity Assay; C20301, Invitrogen). Data shown represent the LDH activity of compound-treated wells, relative to that of paired controls.

Immunohistochemistry

Epithelial monolayers were fixed in paraformaldehyde (4%) and embedded in paraffin. Immuno-detection of CFTR was performed on 5 μm sections using an affinity-purified polyclonal antibody directed against human CFTR (G449, 1:100), kindly provided by Dr. A.C. Nairn (Yale School of Medicine, New Haven) (19, 20). Sections were incubated with the appropriate fluorescently-tagged secondary antibody, i.e. goat-anti-rabbit IgG (A-11008, Invitrogen), and mounted in Vectashield mounting medium supplemented with 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei (Vector Laboratories). Fluorescence was visualized on a Stellaris 5 low incidence angle upright confocal microscope with a HC PL APO CS2 40x/1.30 oil objective (Leica Microsystems).

Western blot analysis

To assess phosphorylation of p38 MAPK, organoid-derived ductal epithelial cells were cultured in 12-well cell culture plates, and were incubated with POA, OA, and/or ethanol in Meyler solution (30 min; 37°C; 5% CO_2). Subsequently, cells were lysed in NaCl (150 mmol/L), Tris/HCl pH 7.6 (25 mmol/L), Triton X100 (1%), sodium deoxycholate (1%), sodium dodecyl sulfate (0.1%), NaF (5 mmol/L), Na_3VO_4 (3 mmol/L), supplemented with a protease inhibitor cocktail (Thermo Fisher Scientific). Lysates were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membrane. Phospho-p38 MAPK (Thr180/Tyr182) was detected using antibody #4511 (1:1000; Cell Signaling Technology), and total p38 MAPK was detected using antibody sc-7972 (1:500; Santa Cruz Biotechnology). Primary antibodies were visualized using fluorescently labeled secondary antibodies (Licor Biotech) and an Odyssey imaging system (Licor Biotech).

Statistical analysis

Data are presented as mean ± SD. Differences between means were statistically analyzed by applying Student's t test or ANOVA (in case >2 experimental groups were compared), as detailed in the figure legends. To control for multiple comparisons in ANOVA, Dunnett's correction was applied.

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Chapter 8

Per-oral pancreatoscopy-guided pancreatic duct biopsy collection in chronic pancreatitis patients allows growth of ductal organoids suitable for fluid and ion transport measurements

Dora Angyal, Erdene Baigal, Tessa A. Groeneweg, Layla Moesker, Willem J. Lammers, Pieter-Jan F. de Jonge, Maikel P. Peppelenbosch, Marco J. Bruno, Marcel J.C. Bijvelds

Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

In preparation

Part II

Synopsis

Dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel has been associated with both genetic and acquired forms of chronic pancreatitis (CP). To assess pancreatic CFTR function in patients diagnosed with CP, we established ductal organoids (PDOs) from biopsies, acquired by per-oral pancreatoscopy.

Abstract

Background & Aims: Dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel has been associated with both genetic and acquired forms of chronic pancreatitis (CP). Access to pancreatic tissue of pancreatitis patients is limited. We cultured organoids from ductal biopsies (PDOs) to assess pancreatic CFTR function in people with CP.

Methods: Biopsies of the main pancreatic duct were obtained during per-oral pancreatoscopy from subjects with CP. PDOs were cultured from biopsies, and gene expression and protein localization were evaluated by RT-qPCR and immunofluorescent staining, respectively. Anion transport was assessed in epithelial monolayers cultured from PDOs, and the effect of the CFTR modulator combination elexacaftor-tezacaftor-ivacaftor (ETI) on ductal fluid secretion was assessed by monitoring PDO swelling.

Results: Pancreatic duct biopsies from 2 out of 7 patients sampled allowed establishment of PDOs that expanded over multiple passages. PDOs contained ductal, epithelial markers (KRT19, KRT7, SOX9, HNF-1β, ECAD) and showed polarized localization of the tight junction protein ZO-1 and CFTR. Cells expressed ion transporters involved in chloride and bicarbonate secretion, including *CFTR, ANO1, SLC26A6 (PAT1)* and *SLC4A4 (NBCe1)*. Consistent with CFTR and ANO1 expression, epithelial monolayers displayed cAMP- and calcium-dependent anion secretion. In one out of two PDO lines tested, ETI treatment enhanced fluid secretion.

Conclusions: Pancreatic duct biopsy allows growth of PDOs that recapitulate the ion transport properties of pancreatic ducts. We propose that, after further optimization, this technique may be exploited to study pancreatic ductal electrolyte and fluid secretion in patients, and to explore precision medicine in CP. Future technical improvements are likely to further facilitate access to this unique material.

Keywords: Per oral pancreatoscopy (POP), CFTR-RD, ETI, organoid, pancreatitis

Introduction

Chronic pancreatitis (CP) is a progressive fibroinflammatory disease of the acinar and ductal epithelia of the exocrine pancreas. CP often manifests with recurrent flares of intense abdominal pain, and may lead to ductal dilatation and calcification, parenchymal atrophy, exocrine and endocrine pancreatic insufficiency, and cancer (1-3). Multiple metabolic and environmental factors are thought to play a role in the complex etiology of CP. Prominent risk factors are biliary reflux, alcohol consumption, tobacco smoking, metabolic abnormalities, and autoimmune disease (1-3). Still, in many cases no direct cause can be identified. Currently, no specific therapy is available, and management of CP relies on supportive care.

Mutations in a distinct set of genes have been shown to confer an increased risk of developing CP (4, 5). Many of these genes appear to play an intricate role in the processing of zymogens produced by acinar cells, and mutations in these genes are thought to increase the risk of tissue injury by promoting premature, intra-pancreatic activation of trypsin (4-6). One of the genes associated with CP is *CFTR*, which encodes the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial anion channel that is critically involved in the production of a bicarbonate-rich, alkaline pancreatic juice (7, 8). Those carrying loss-of-function mutations on both *CFTR* alleles develop cystic fibrosis (CF), a complex, multi-system disease that affects ion and fluid transport across epithelia of, mainly, the airways and gastrointestinal tract. In most people with CF (pwCF), CFTR dysfunction leads to a complete loss of exocrine pancreatic function; only CF alleles that allow some residual CFTR function cause a milder pancreatic disease phenotype. Still, this exocrine pancreatic sufficient group has a high risk of developing pancreatitis (9, 10).

Apart from CF causing alleles, there are numerous further *CFTR* variants that do not cause CF, but are nevertheless thought to confer an increased risk of developing recurrent acute pancreatitis or CP (11-13). Indeed, an estimated 30% of all idiopathic cases of CP carry mutations on at least one of their *CFTR* alleles (10, 14). However, because assessment of CFTR function is not routinely performed in those suspected of (idiopathic) CP, the role of such variants in the development of pancreatic disease is often overlooked (15-17). Most of these variants may only subtly affect channel function, and in carriers of such alleles, haploinsufficiency becomes apparent only in combination with another genetic disease modifier and/or after exposure of the pancreas to a metabolic or environmental stressor (4, 18). When CFTR dysfunction is confirmed, e.g. by assessing biomarkers of CFTR function such as sweat chloride concentration, carriers of such variants are increasingly diagnosed with CFTR-related disease (CFTR-RD) (17). Some evidence indicates that CFTR dysfunction in CP may not only have a genetic basis, but may also be acquired. Typical stressors like ethanol, tobacco smoke and bile acids were proposed to reduce the activity of CFTR in ductal

epithelium (19). The resulting impairment of CFTR-dependent ductal anion and fluid secretion is thought to play a central role in triggering or exacerbating the inflammatory response.

The introduction of small-molecule drugs that enhance the activity of mutant CFTR has dramatically improved the disease prognosis and quality of life of pwCF (20). By improving co-/posttranslational protein folding and trafficking of the nascent protein, *correctors* increase the amount of CFTR at the cell surface, whereas *potentiators* improve channel gating. These CFTR modulators have been shown to also improve symptoms and reduce hospitalizations due to pancreatitis in exocrine pancreatic sufficient pwCF (21, 22). Recent evidence indicates that CFTR modulators also reduce the number of acute pancreatitis episodes in individuals with CFTR-RD (23).

Presently, there are no assays available to directly assess ductal CFTR function. Consequently, in individual CP cases, it is generally uncertain to what extent CFTR dysfunction is involved in the development of pathology, and whether CFTR modulators could ameliorate disease symptoms. To address these issues, we aimed to develop a method to establish pancreatic ductal organoids (PDOs) from biopsies obtained during per-oral pancreatoscopy in CP subjects. The pancreatic ductal phenotype of PDOs was established, epithelial anion and fluid transport was assessed in PDO-based assays, and the effect of the CFTR modulator combination elexacaftor-tezacaftor-ivacaftor (ETI) on CFTR-dependent fluid secretion was investigated.

Results

Per-oral pancreatic duct biopsy allows establishment of organoids

To investigate whether biopsies harvested from the main pancreatic duct can be used to establish primary, ductal, epithelial cell cultures, twelve individuals diagnosed with CP were approached. All those included had one or more pancreatic duct stones in the head or the neck region of the pancreas, and were scheduled to undergo per-oral pancreatoscopy and electrohydraulic lithotripsy (EHL). Four patients were eventually excluded from the study: in two, pancreatoscopy was judged unnecessary during the endoscopic retrograde cholangiopancreatography procedure (ERCP), while in two further individuals the presence of strictures precluded the procedure. The final study population included six men and two women. The risk factor excess alcohol usage was present in 3 out of 8 cases (Table 1). No evident risk factors were identified in the remaining individuals.

Table 1. Characteristics of patients (n=8)

Demographics	
Female sex, n (%)	2 (25)
Age at inclusion (years), median (interquartile range)	52 (46-62)
Age at onset of pancreatitis (years), median (interquartile range)	49 (38-61)
Clinical characteristics	
Etiology, n (%)	Excess alcohol usage: 3 (37.5) Idiopathic: 5 (62.5)

Using a SpyBite™ Max single-use biopsy forceps, pancreatic duct biopsies were obtained from seven out of eight patients undergoing EHL (Fig. 1, Fig. 2A,B). Our protocol allowed for the sampling of two biopsies in each individual. The diameter of the biopsies collected ranged between circa 0.5-2 mm. In two cases, only little material was collected, and the few cells isolated failed to expand and self-organize into organoids. Cell clusters isolated from the biopsies of the other five patients formed spheroidal structures with an apparently fluid-filled central lumen when cultured in an extracellular matrix (Fig. 2C). However, a persistent microbial contamination precluded further expansion of three of these samples. Perhaps unexpectedly in view of the tissue origin, in the present cohort, contamination was found only in these pancreatic primary cultures, but in none of the eight duodenal lines established. Attempts to rescue these pancreatic cultures by the addition of vancomycin to the culture medium and by increasing the concentration of amphotericin proved unsuccessful, and this regimen

may have further reduced the viability of the cultures. Self-renewing, ductal cell cultures were established from biopsies of two individuals. These pancreatic ductal organoid (PDO) cultures could be expanded over multiple passages (>4), allowing phenotyping and biobanking (Fig. 1). Subject P1 was 19 years old at the time of biopsy sampling, with an initial pancreatitis episode, classified as idiopathic, at age 17. No risk factors like smoking or alcohol consumption were evident. Subject P2 was 49 years old at the time of biopsy sampling, had a history of excess alcohol usage and tobacco smoking, and suffered an initial pancreatitis episode at age 48.

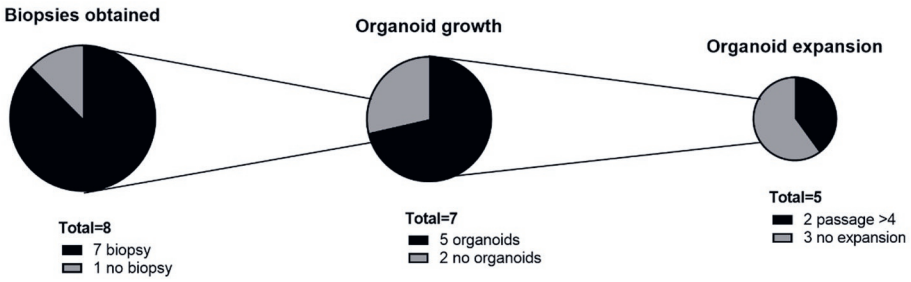


Figure 1. Flowchart from patient inclusion to human pancreatic organoid expansion. Pancreatoscopy was performed in eight patients. In seven of these, pancreatic duct biopsies were obtained. From five patients organoids were established. Two of the established organoid lines were expanded over >4 passages.

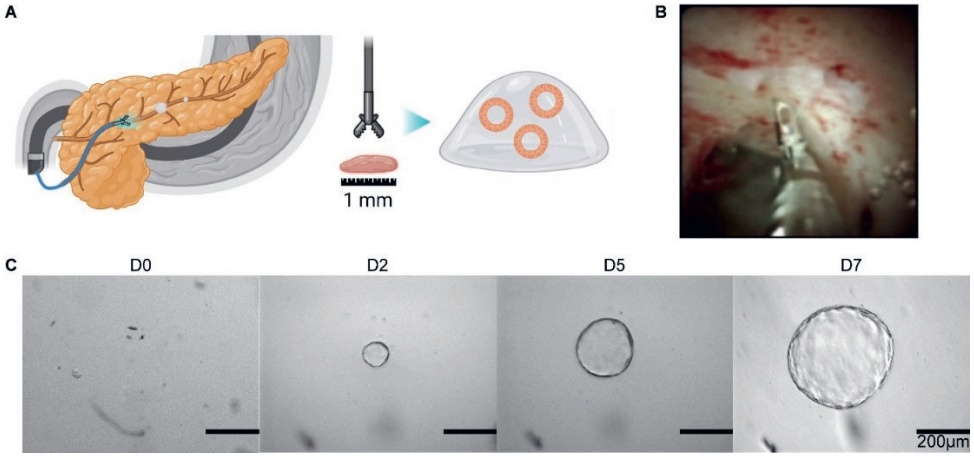


Figure 2. Organoid culture from per-oral pancreatic duct biopsies of CP patients. (A) Schematic representation of PDO culture establishment; from per-oral pancreatoscopy, to main pancreatic duct biopsy and extracellular matrix embedded organoids. (B) SpyBite™ Max Biopsy Forceps obtaining a biopsy from the main pancreatic duct. (C) Representative brightfield micrographs of organoids obtained from P1 at 0, 2, 5 and 7 days after seeding isolated cells in an extracellular matrix. Scale bar: 200 µm.

Genetic analysis identifies CFTR variants in P1 and P2

The sequence of the coding and the short flanking intronic regions of nine pancreatitis-associated genes was assessed by next-generation sequencing. This demonstrated that both subject P1 and subject P2 carried a *CFTR* variant that has previously been linked to pancreatitis (Table 2). Subject P1 was a heterozygous carrier of the p.Arg75Gln (c.224G>A) *CFTR* variant, which has an estimated allele frequency in the general population of 3.0% (https://gnomad.broadinstitute.org). Although it does not cause CF, a study of a North American cohort indicated that carriers of this variant are at an increased risk of developing pancreatitis (13). However, studies on German and French cohorts failed to confirm its overrepresentation in pancreatitis cases (14, 24). Apart from this *CFTR* variant, P1 carried the common synonymous *CTRC* variant p.Gly60Gly (c.180C>T; estimated allele frequency in general population: 9.9%). In heterozygous carriers, this variant has been shown to modestly increase the risk of developing pancreatitis relative to the c.180CC genotype (allelic odds ratio: 1.9) (25). In the present study population of eight patients, one other subject carried this variant.

Subject P2 was a heterozygous carrier of the CF-causing *CFTR* variant p.Phe508del (c.1521_1523del). This in frame deletion impairs proper folding of newly formed CFTR protein, leading to premature proteasomal degradation and reduced insertion into the plasma membrane. Heterozygous carriers do not develop CF, but are at increased risk of developing a CFTR-RD (4, 16, 24, 26).

We also investigated the polypyrimidine tract, located immediately 5' of exon 10 in the *CFTR* locus (IVS-9 poly-TG/poly-T). The T[5] allele has been shown to affect exon splicing, and is associated with CFTR-RD (16, 27). This analysis demonstrated that all of the eight subjects in the present study group exclusively carried T[7] and/or T[9] alleles.

Other than the *CFTR* and *CTRC* variants listed above, we detected a rare *CPA1* variant in one subject, p.Arg54Arg (c.160C>A; allele frequency: 0.003%). The clinical significance of this synonymous mutation is unclear. No rare and/or established disease-associated variants were observed in *CASR*, *CTSB*, *PRSS1*, *PRSS2*, *SPINK1*, or *TRPV6*.

Table 2. *CFTR* and *CTRC* genetic variant analysis. Coding and adjacent short intronic sequences, including the polypyrimidine tract in intron 9 of the *CFTR* locus, were probed for the presence of established pancreatitis-associated mutations, or rare variants.

Subject	***CFTR***		***CTRC***
		IVS-9 poly-TG/poly-T	
P1	p.Arg75Gln (c.224G>A) / -	TG[11]-T[7] / TG[12]-T[7]	p.Gly60Gly (c180C>T) / -
P2	p.Phe508del (c.1521_1523del) / -	TG[8]-T[7] / TG[10]-T[9]	- / -

Organoids and epithelial monolayers cultured from pancreatic duct biopsies express ductal markers

Gene expression in PDOs was assessed by qPCR. For comparison, we also included PDOs derived from healthy cancer-adjacent tissue (HP) in our analysis. Organoid cells expressed many genes which are characteristic of the pancreatic ductal epithelium, including keratin 19 (*KRT19*), secreted phosphoprotein 1 (*SPP1*), CFTR (*CFTR*) and aquaporin (*AQP1*) (Fig. 3A). Transcript levels of genes typically expressed in other principal pancreatic cell types were generally lower, although expression of serine protease 1 (*PRSS1*), typically found in acinar cells, was also observed (28). Crucially, we also detected transcripts encoding proteins involved in ductal anion secretion (29). Cells expressed the principle cellular bicarbonate and chloride uptake mechanisms, i.e. the sodium-coupled cotransporters NBCe1 (*SLC4A4*) and NKCC1 (*SLC12A2*), respectively, and the sodium proton exchanger NHE1 (*SLC9A1*) which, through proton extrusion, contributes to intracellular alkalization (Fig. 3A). Further, we detected expression of the chloride-bicarbonate exchanger PAT1 (*SLC26A6*) a key anion transport mechanism involved in ductal bicarbonate secretion. We also detected low levels of another chloride-bicarbonate exchanger, DRA (*SLC26A3*). Apart from the cAMP-regulated CFTR anion channel, we noted that the calcium-dependent chloride channel (CaCC) anoctamin 1/TMEM16A (*ANO1*) was expressed in PDOs. When seeded on a permeable substrate, PDO-derived cells formed an epithelial monolayer within 1-2 weeks. The gene expression pattern observed in these monolayers was similar as in PDOs (Fig. 3B).

We used immunocytochemistry to characterize the cellular partitioning of principal marker proteins in PDOs and epithelial monolayers (Fig. 4). Cells contained structural proteins KRT19 and KRT7, and we detected nuclear localization of the transcription factors SOX9 (*SOX9*) and HNF-1β (*HNF1B*). These ductal, epithelial markers are shared between ducts of the ventral pancreas and extrahepatic cholangiocytes, which have a common embryologic origin (28, 30). Additionally, e-cadherin (ECAD, *CDH1*) was located at the lateral aspect of the cells, consistent with the presence of adherens junctions. We also detected the tight junction protein zonula occludens 1 (ZO-1, *TJP1*) at the junction between adjacent cells, and close to their apical pole. CFTR was detected exclusively in the lumen-facing plasma membrane, consistent with the formation of a polarized epithelium.

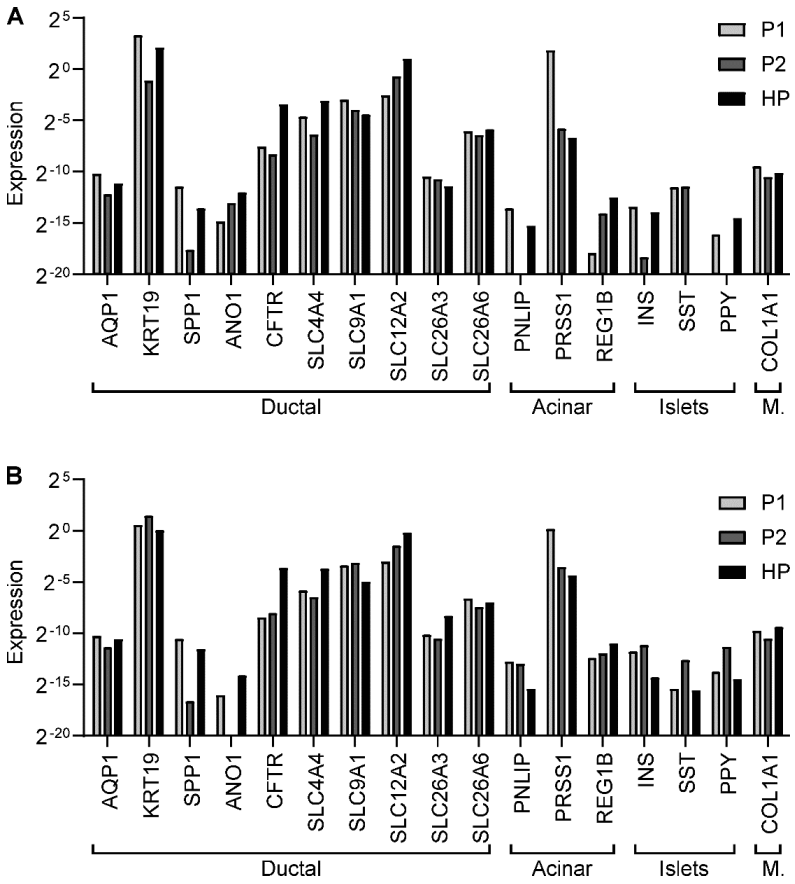


Figure 3. Expression of ductal, acinar, islet and mesenchymal (M.) markers in CP (P1, P2) and HP PDOs and PDO-derived monolayers. Gene expression was assessed by RT-qPCR in (A) PDOs and (B) PDO-derived monolayers.

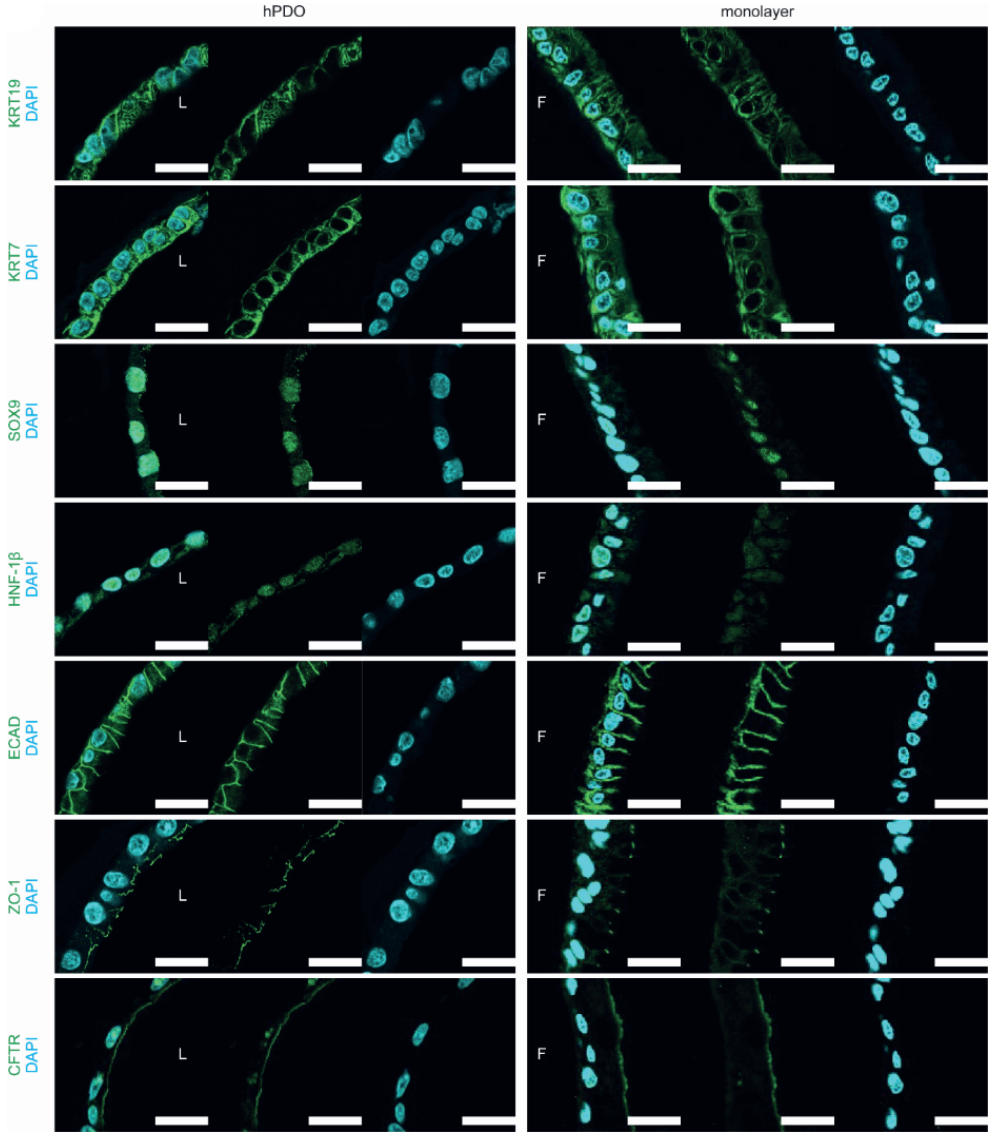


Figure 4. Localization of ductal markers in PDOs and epithelial monolayers. Localization of marker proteins was assessed by immunocytochemistry in PDOs (left panel) and PDO-derived monolayers (right panel). Nuclei were stained with DAPI. Orientation of central lumen (L) and Transwell insert (F) indicated in overlay (left) of separate channels (middle and right). Scale bars: 25 μm.

PDO-derived epithelial monolayers display CFTR- and CaCC-mediated anion secretion

Vasoactive intestinal peptide (VIP) stimulates pancreatic ductal fluid secretion through activation of Gs-coupled receptors that trigger cAMP production and activation of the cAMP-dependent protein kinase (PKA) (8). VIP stimulated anion secretion in HP epithelial monolayers (Fig 5). VIP also triggered an Isc response in P1 and P2 monolayers, but the amplitude of the response was smaller than in HP monolayers. The Isc response was not increased further by combined exposure to the adenylyl cyclase/cAMP agonist forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). The response was strongly attenuated by the CFTR inhibitor BPO-27.

Addition of UTP to the lumen-facing bath provoked a transient anion secretory response in PDO-derived epithelial monolayers (Fig. 5). This indicated the presence of purinergic receptors that, through an increase in cellular Ca^{2+}, activated a CaCC (31). The effect of UTP was evaluated in the presence of BPO-27, excluding a contribution of CFTR to the observed response. Consistent with CaCC activation, the ANO1 blocker TMEM16-A01 blunted the response to UTP. The anion secretory response to UTP was of similar amplitude in CP *vs*. HP monolayers.

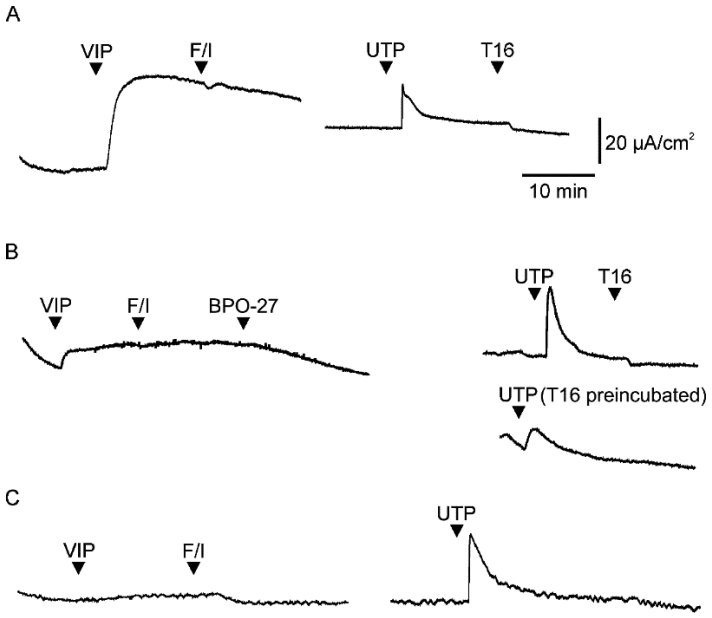


Figure 5. CFTR- and CaCC-mediated anion secretion in PDO-derived epithelial monolayers. Epithelial anion transport was assessed by short-circuit current (Isc) measurement in PDO-derived monolayers of (A) HP, (B) P1, or (C) P2. VIP triggered an Isc response that was sensitive to the CFTR inhibitor BPO-27. Addition of forskolin and IBMX (F/I) did not further stimulate the secretory response. The Isc response elicited by UTP, applied in the presence of BPO-27, was attenuated by the CaCC inhibitor T16Ainh-A01 (T16).

Part II

Effect of CFTR modulators on CFTR-dependent fluid secretion in CP PDOs

Next, we asked whether CFTR modulators could improve CFTR function in pancreatic ductal epithelium of CP patients. We assessed CFTR-dependent fluid secretion across ductal epithelium by measuring the increase in PDO size after forskolin stimulation. Quantification of forskolin-dependent swelling is a technique routinely used to evaluate CFTR function in intestinal organoids (32, 33). Both PDOs from HP and P2, but not from P1, displayed fluid accumulation when exposed to forskolin (Fig. 6A-C). ETI treatment partially restored forskolin-dependent fluid accumulation in PDOs of P1. The potentiator ivacaftor, in the absence of the correctors elexacaftor and tezacaftor, tended to also increase forskolin-dependent fluid transport in P1, but its effect was less pronounced than of the triple combination. These data align with a previous study demonstrating that ETI treatment markedly enhances the activity of Arg75Gln-CFTR expressed in Fisher Rat Thyroid (FRT) cells (34). In P2, ETI tended to enhance the fluid secretory response, consistent with rescue of Phe508del-CFTR function, but the apparent difference did not reach statistical significance. Ivacaftor, in the absence of correctors, did not increase forskolin-dependent fluid accumulation in PDOs from P2.

Because both P1 and P2 carried *CFTR* variants, we hypothesized that a functional defect would also be apparent in other CFTR-expressing epithelia. Therefore, we tested the effect of modulator treatment on fluid accumulation in duodenal organoids derived from these CP subjects. This showed that, in intestinal organoids of both individuals, forskolin robustly stimulated fluid accumulation, and neither ivacaftor nor ETI further enhanced fluid accumulation, suggesting intestinal anion and fluid secretion was not affected (Fig. 6D,E).

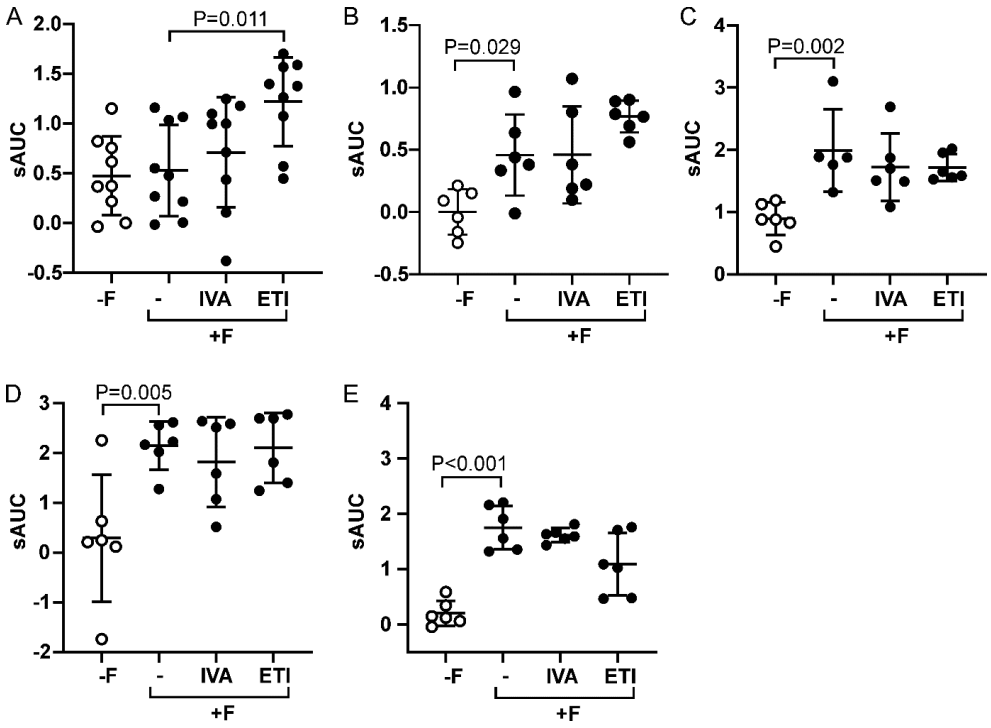


Figure 6. Effect of CFTR modulators on forskolin-dependent swelling in PDOs and intestinal organoids. After organoids were treated with elexacaftor and tezacaftor or vehicle (DMSO) for 20h, forskolin (F) was added in the presence or absence of ivacaftor (IVA) or elexacaftor-tezacaftor-ivacaftor (ETI) to trigger CFTR-dependent organoid swelling. The cumulative increase in organoid size was assessed over a 60 min period, and was expressed as the signed area under the curve (sAUC). Forskolin-dependent organoid swelling in (A) P1 PDOs, (B) P2 PDOs, (C) HP PDOs, (D) P1 intestinal organoids, and (E) P2 intestinal organoids. Each data point represent one technical replicate. Statistical analysis: ANOVA.

Discussion

In this study, we generated PDOs from biopsies that were obtained through per-oral sampling of the main pancreatic duct of CP patients that underwent EHL. The resulting cultures expressed genes that are characteristic of the pancreatic ductal epithelium. Epithelial monolayers cultured from PDO-derived cells displayed both CFTR- and CaCC-mediated anion secretion upon stimulation of VIP- or purinergic receptors, respectively. CFTR-dependent fluid transport in PDOs cultured from the tissue of CP subjects carrying *CFTR* variants was low, compared to non-CP PDOs, but was enhanced by treatment with CFTR modulators in one out of the two lines investigated. This proof-of-principle study shows that that PDOs can be cultured from biopsies of the main pancreatic duct. While further optimization of the sampling and cell culture procedures is indicated, we propose that PDOs may offer a viable platform to study ductal ion transport physiology and drug responses in the context of CP.

In the presently investigated two CP cases, CFTR activity in pancreatic ductal epithelium was low, compared to control tissue. Both *CFTR* variants detected in these subjects have previously been linked to an increased risk of pancreatitis (13, 24, 26). We did not find disease causing mutations on the second allele carried by these subjects. This suggests that these variants, even *in trans* of a wildtype allele, impair ductal anion and fluid secretion. In contrast, fluid transport across the intestinal epithelium of these subjects was unaffected. It has been demonstrated before that obligate carriers of CF alleles display normal intestinal, CFTR-dependent anion and fluid secretory responses, most plausibly because uptake across the basolateral plasma membrane constitutes the rate-limiting step in intestinal anion secretion (35, 36). Our direct measurement of pancreatic CFTR function in these carriers of variant *CFTR* alleles indicates that in pancreatic ductal epithelium, the capacity for CFTR-mediated efflux across the apical plasma membrane is more critical. Consistent with this notion, pancreatitis is a prominent example of CFTR-RD, a clinical condition associated with moderate CFTR dysfunction, which (other than CF) can also affect those carrying only a single pathogenic *CFTR* allele (16). The fact that two individuals in this cohort of eight carried such alleles is in agreement with previous estimates of the incidence of *CFTR* variants in idiopathic CP (10).

Apart from by a genetically conferred defect, i.e. a mutation in *CFTR*, ductal anion and fluid secretion is thought to be attenuated by exposure of the epithelial cells to environmental stressors and inflammation mediators (19). It was proposed that such stressors impair ductal anion and fluid secretion by altering the subcellular partitioning of CFTR protein in the ductal epithelium (37-39). Because our assays were performed on PDO cultures that had been maintained and sub-cultured over several weeks, it seems implausible that exogenous factors affected their fluid secretory response, unless through epigenetic regulation. In addition, it is conceivable that ion transport

mechanisms operating upstream of CFTR, or other elements essential to the anion secretory response, are (also) downregulated. In this context, it is important to note that UTP-dependent, CaCC-mediated, anion secretion was comparable between CP and HP epithelial monolayers, indicating that the observed defect is restricted to the CFTR-dependent pathway of anion and fluid secretion.

In our assays, ETI treatment enhanced forskolin-dependent fluid secretion in PDOs of P1, and, more tentatively, also P2. ETI appeared more effective than IVA alone, indicating that the improvement in the fluid secretory response must, in part, be attributed to an increase in CFTR cell surface expression. ETI treatment is known to markedly improve trafficking and channel function of Phe508del-CFTR, but has also been demonstrated to enhance protein maturation and activity of the Arg75Gln-CFTR variant carried by P1 (34). Apart from their effect on CFTR, modulators may enhance CFTR-dependent anion secretion indirectly, by modulation of other ion transport mechanisms involved in the anion secretory response, including potassium channels (40).

As few other indications for performing per-oral pancreatoscopy exist, our study did not include PDOs that were cultured from the main pancreatic duct of individuals not afflicted by CP. As a substitute, we included PDOs generated from resected non-CP, non-cancerous pancreatic tissue in our study. Although the gene expression profile, as well as their CaCC activity, were similar, it is conceivable that the CP and HP PDOs stem from separate sections of the ductal tree, with inherently different ion transport properties. *CFTR* was found to be expressed along the entire length of the ductal tree, but its exact role in bicarbonate secretion differs subtly between different anatomical sections (29, 41). Computational modeling has indicated that, whereas in proximal sections CFTR may facilitate bicarbonate secretion via members of the SLC26 family of chloride-bicarbonate exchangers, in the distal part, channel-mediated bicarbonate secretion must predominate (42). CFTR may transport bicarbonate directly, but has also been proposed to control CaCC activity by regulating luminal ATP release (43, 44).

In this proof-of-concept study, only a small number of patients was included, and the culture of PDOs suitable for drug testing was achieved in only 2 out of 7 instances where biopsies were obtained. Consequently, further optimization of the protocol is needed to ensure that viable cultures can be established for phenotyping and drug testing. A critical step amenable to further optimization is the sampling of the biopsies. Based on our present experience, we surmise that the likelihood of successful organoid establishment critically depends on the size of the biopsies that are obtained. The smaller specimens sampled only included the more superficial layers of the ductal mucosa, and we conjecture that these contain insufficient adult stem cells for the initiation of organoid cultures. We anticipate that further technical advances, e.g. in the design of the biopsy forceps and the optical system, may help to improve sampling

consistency, and increase the overall success rate of the procedure. A second critical aspect is the germ-free culturing of the isolated cells. We found that microbial contamination of ductal biopsies often persists, despite the inclusion of a broad spectrum of antibiotics/-myotics in the media used for cell isolation and culture. In contrast, germ-free organoid lines could readily be cultured from intestinal biopsies of all individuals sampled, suggesting that the pancreatic cultures are particularly prone to microbial contamination. Once manifest, microbial colonization proved difficult to eradicate. Consequently, the success of the procedure principally relies on preventative measures, and it is imperative that biopsies are extensively and repeatedly rinsed to reduce the microbial load in the initial stages of culture establishment. Furthermore, for similar reasons, we recommend frequent (daily) refreshment of culture media during the early stages of organoid establishment (45).

In summary, we demonstrate that biopsies can be obtained from the main pancreatic duct that allow the establishment of primary epithelial cell cultures, which recapitulate the ion transport properties of pancreatic ductal epithelium. We propose that this model may be used to improve treatment of CP, which now principally relies on supportive care. Finally, our findings imply that CFTR modulators, by improving ductal anion and fluid secretion, may ameliorate disease symptoms in some individuals with CP.

Methods

Selection of patients

Patients were enrolled between June 2021 and November 2024, at the Erasmus MC University Medical Center, Rotterdam, the Netherlands, an academic, tertiary referral center for hepatopancreaticobiliary diseases. Inclusion criteria were: patient undergoing per-oral pancreatoscopy within the framework of regular treatment, aged ≥18 years. Written informed consent was obtained from each participant. The study was conducted according to the guidelines in the Declaration of Helsinki and was approved by the medical ethics committee of the Erasmus MC (MEC-2021-0737). The medical records of the enrolled subjects were retrospectively reviewed. Demographical and clinical data (e.g. medication use, alcohol consumption and tobacco smoking, disease symptoms, prior episodes of pancreatitis), and findings of imaging studies (e.g. duct strictures or dilatation) were collected.

Pancreatic ductal organoid cultures derived from healthy cancer-adjacent tissue were obtained from the Foundation Hubrecht Organoid Biobank (Utrecht, The Netherlands), as specified by material transfer agreements 21/034 and 25/054.

Genetic variant analysis

Genomic DNA was extracted from whole blood samples using a genomic DNA isolation kit (ReliaPrep; Promega). Sample quality control, library preparation and whole exome sequencing was performed by Macrogen Europe. Libraries were prepared using the Twist Exome 2.0 library kit (Twist Bioscience), and sequencing was performed on the NovaSeq X platform (sequencing depth: 50x; Illumina). Reads were aligned to a reference genome (HG38; Genome Reference Consortium) using the HISAT2 software module (v. 2.2.1), on the Galaxy platform (https://usegalaxy.eu). Coding and adjacent short intronic sequences of 9 genes were scanned for the presence of established pancreatitis-associated mutations, as well as rare variants (reported allele frequency in the Genome Aggregation Database: <1%; https://gnomad.broadinstitute.org). The genes examined were: *CASR* (ENST00000639785.2), *CFTR* (ENST00000003084.11), *CPA1* (ENST00000011292.8), *CTRC* (ENST00000375949.5), *CTSB* (ENST00000353047.11), *PRSS1* (ENST00000311737.12), *PRSS2* (ENST00000539842.6), *SPINK1* (ENST00000296695.10), and *TRPV6* (ENST00000359396.9).

Tissue collection

Endoscopy was performed with a video duodenoscope (ED34-i10T2; Pentax Medical), and for pancreatoscopy a digital single-operator cholangiopancreatoscopy system (SpyGlass DS Direct Visualization System; Boston Scientific) was used. Specifically, a SpyScope™ DS II catheter (M00546610; Boston Scientific) was advanced either over a

guidewire (diameter: 0.035 inch), or freehand, into the main pancreatic duct. To facilitate the introduction of the pancreatoscope, pancreatic sphincterotomy was performed, or, if necessary, a pre-existing sphincterotomy was extended. Two biopsies were taken under direct vision using a SpyBite™ Max single-use biopsy forceps (M00546470; Boston Scientific). EHL was performed as described previously (46). In addition, two duodenal biopsies were obtained using a single-use standard biopsy forceps (diameter: 2.8 mm).

Organoid culture

Organoids were generated from pancreatic ductal biopsies by adapting established protocols (45). After sampling, biopsies were collected and stored for up to 2 h in ice-cold Ad-D/F+++: advanced DMEM/F12 (Gibco), GlutaMAX (2 mmol/L; Gibco), HEPES (10 mmol/L; Gibco), primocin (50 µg/mL; InvivoGen), penicillin (100 U/mL; Thermo Fisher Scientific), streptomycin (100 µg/mL; Thermo Fisher Scientific) and Y-27632 (10 µmol//L; R&D Systems). Before further processing, biopsies were transferred to Ad-D/F+++ supplemented with amphotericin B (0.25 µg/mL; Thermo Fisher Scientific) and vancomycin (100 µg/mL; Brunschwig Chemie), and washed by repeated aspiration in a 10 mL serological pipette. After centrifugation (5 min, 150 *g*, 4°C), the tissue was incubated (20 min; 37°C) on a shaking platform in digestion buffer (5 mL): Hank's balanced salt solution (HBSS), collagenase type II (1 mg/mL; Thermo Fisher Scientific), Y-27632 (10 µmol/L), trypsin inhibitor (100 µg/mL; Gibco), glycerol (5%). Tissue dissociation was aided by repeated aspiration through a 1 mL micropipette tip at 5 min intervals. When cell clusters containing approximately 10 cells had formed, fetal bovine serum (10%; Gibco) was added. Cell clusters were collected by centrifugation (5 min, 300 *g*, 4°C), resuspended in Ad-D/F+++ supplemented with amphotericin B and vancomycin (see above), and washed twice. After centrifugation (5 min, 300 *g*, 4°C), cell were finally collected in Ad-D/F+++, and embedded in extracellular matrix (Matrigel; Corning). The embedded cells were cultured in *pancreatic normal medium*, supplemented with forskolin (10 µmol/L), as specified by Driehuis *et al*. (45). During the first seven days after seeding, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL) were added to the culture medium. The culture medium was further supplemented with Y-27632 (10 µmol/L) during the first two days after seeding and during the first two days after passaging. Intestinal organoids were cultured from mucosal biopsies as described elsewhere (33).

For culture of epithelial monolayers, organoids were enzymatically dissociated in TrypLE Express (1x; Thermo Fisher Scientific). After enzyme activity was quenched by addition of ice-cold Ad-D/F+++, cells were collected by centrifugation, resuspended, and seeded ($6 \cdot 10^5/cm^2$) on permeable inserts (Transwell #3470; Corning) that had been pretreated with Matrigel diluted in phosphate buffered saline (PBS; 1:20; 0.2 mL/cm^2) for 2 h at 37°C. Culture medium was as for extracellular matrix-embedded

organoids, except that, to suppress CFTR activity, the adenylyl cyclase agonists prostaglandin E2 (PGE2) and forskolin were removed from the culture medium. Cells were cultured until a confluent monolayer was obtained (10-14 days).

Gene expression

Organoids were collected in ice-cold PBS and extracellular matrix was removed by washing twice. RNA was extracted from organoids and organoid-derived epithelial monolayers using the NucleoSpin RNA kit (Macherey–Nagel), and synthesis of cDNA was performed using PrimeScript RT Enzyme Master Mix (Takara Bio). Quantitative reverse transcriptase PCR (primer sequences shown in Table 3) was performed in SYBR Green I Master mix (Thermo Fisher Scientific) using the StepOnePlus RT-PCR platform (Applied Biosystems, Thermo Fisher Scientific). Median values of assays performed in triplicate were used to determine gene expression levels relative to *YWHAZ*.

Table 3 Primer sequences used for RT-PCR

Gene	Accession number	Forward	Reverse
KRT19	NM_002276.5	ACCAAGTTTGAGACGGAACAG	CCCTCAGCGTACTGATTTCCT
SPP1	NM_001040058.2	GAAGTTTCGCAGACCTGACAT	GTATGCACCATTCAACTCCTCG
CFTR	NM_000492.3	TGGATCGCTCCTTTGCAAGT	AAGTCCACAGAAGGCAGACG
AQP1	NM_198098.4	GGTCAAGCCTCTTATGGGGG	CTAGCAGGTGGGTCCCTTTC
PRSS1	NM_002769.5	GGCTCCCTCATCAACGAACA	CTTCCTGTCGTATTGGGGGT
PNLIP	NM_000936.4	TCAGTCGGCGTTCGGTTAC	TGTGATGCGTCCAATGGTCC
REG1B	NM_006507.4	CCTCAAGTGGACAAGGCACT	TGATTCGGGGATTAGGCAGC
INS	NM_000207.3	GCAGCCTTTGTGAACCAACAC	CCCCGCACACTAGGTAGAGA
SST	NM_001048.4	CCCAGACTCCGTCAGTTTCT	AAGTACTTGGCCAGTTCCTGC
PPY	NM_002722.5	CCTGCGTGGCTCTGTTACTAC	CCTGGTCAGCATGTTGATGTATC
COL1A1	NM_000088.4	GTGCGATGACGTGATCTGTGA	CGGTGGTTTCTTGGTCGGT
ANO1 (TMEM16A)	NM_018043.7	GACCTCACGGGCTTTGAAGA	GCCATGGCTGTCTTAACCCT
SLC12A2 (NKCC)	NM_001046.3	TGGGTCAAGCTGGAATAGGTC	ACCAAATTCTGGCCCTAGACTT

SLC26A3 (DRA)	NM_000111.3	CAGTGGCCTTTTCAGTTGCC	GGGCAGTACTCCCAGCAAAT
SLC26A6 (PAT)	NM_022911.3	GTCCGAGGCTATACCACAGC	CTGCTCAGATGGAGGCCAAA
SLC9A1 (NHE1)	NM_003047	ACCACGAGAACGCTCGATTG	ACGTGTGTGTAGTCGATGCC
SLC4A4 (NBCe1-B)	NM_001098484.3	CCTCAAGCATGTGTGTGATGA	ACTCTTCGGCACATGGACTC
YWHAZ	NM_003406.4	CCTGCATGAAGTCTGTAACTGAG	GACCTACGGGCTCCTACAACA

Immunocytochemistry

Pancreatic organoids or epithelial monolayers were fixed in paraformaldehyde (4%) and embedded in paraffin. Immuno-detection of ductal proteins was performed on 5 µm sections using antibodies directed against keratin 19 (M0888, 1:100; Agilent Dako), keratin 7 (M7018, 1:100; Agilent Dako), SOX9 (AMAb90795, 1:500; Atlas Antibodies), HNF-1β (GTX129236, 1:250; GeneTex), e-cadherin (#3195, 1:300; Cell Signaling Technology), zonula occludens 1 (21773-1-AP, 1:2000; Proteintech) or an affinity-purified polyclonal antibody directed against human CFTR (G449, 1:100), kindly provided by Dr. A.C. Nairn (Yale School of Medicine, New Haven) (47). Sections were incubated with an appropriate fluorescently-tagged secondary antibody, i.e. goat-anti-mouse IgG (A-11032, Invitrogen) or goat-anti-rabbit IgG (A-11008, Invitrogen), and mounted in Vectashield mounting medium supplemented with 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei (Vector Laboratories). Fluorescence was visualized on a Stellaris 5 low incidence angle upright confocal microscope with a HC PL APO CS2 40x/1.30 oil objective (Leica Microsystems).

Fluid transport in organoids

CFTR-mediated anion secretion stimulates osmotic fluid transport into the enclosed lumen of intestinal organoids, leading to organoid swelling (32, 33). To evaluate fluid secretion, organoids were seeded in 96-well cell culture plates in droplets (3 µL) of extracellular matrix (Matrigel), and cultured for 2-3 days. For PDOs, to suppress CFTR activity, the adenylyl cyclase agonists PGE2 and forskolin were removed from *pancreatic normal medium*. Where appropriate, organoids were cultured (20h) in the presence of elexacaftor (VX-445; 3µmol/L; MedChem) and tezacaftor (VX-661; 3µmol/L; MedChem), or vehicle (dimethyl sulfoxide (DMSO), 0.2%) prior to the assay. Subsequently, cells were incubated with calcein-AM (5 µmol/L, 30 min, Invitrogen), and CFTR-dependent organoid swelling was triggered by the addition of forskolin (10 µmol/L) in the presence or absence of the CFTR potentiator ivacaftor (VX-770; 0.3 µmol/L; Selleckchem). Live-cell imaging was performed using a Leica TCS SP5 confocal microscope (Ex=488 nm, 5x objective), equipped with a cell incubation system (37°C,

5% CO_2; Leica Microsystems). Micrographs were captured at 6 min intervals using LAS AF software (Leica Microsystems). The total area occupied by the fluorescently labeled organoids in each droplet/well (containing approximately 30 organoids) was quantified on the ImageJ platform (https://imagej.net), using a software module that automates image analysis. Data shown represent the cumulative increase in area over 60 min, expressed as the signed area under the curve (sAUC), where negative values denote a decrease in area relative to t = 0 min.

Electrophysiological assessment of epithelial anion transport

Organoid-derived pancreatic ductal epithelial monolayers were mounted in P2302T/P2300-type Ussing chambers (surface area: 0.33 cm^2; Physiologic Instruments, San Diego), and bathed in Meyler solution (mmol/L): 128 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, 10 glucose. Solutions were maintained at 37°C, adjusted to pH 7.3 and gassed continuously with 95% O_2, 5% CO_2. The transepithelial potential difference was clamped at 0 mV (VVC-MC8 module; Physiologic Instruments), and the resulting short-circuit current (Isc) was recorded with a PowerLab 8/35 AD converter and associated software (LabChart 8; AD Instruments).

CFTR activity was stimulated by addition of either vasoactive intestinal peptide (VIP, 50 nmol/L; Bachem), added to the contra-luminal bath, or the combination of forskolin (10 µmol/L) and IBMX (50 µmol/L; Sigma Aldrich; added bilaterally). BPO-27 (20 µmol/L; Bio-Connect) was used to inhibit CFTR activity. UTP (50 µmol/L; Sigma-Aldrich) was added to the luminal bath to stimulate purinergic receptors that couple to calcium-dependent chloride channels (CaCC). UTP responses were assessed in the presence of BPO-27. T16A-inhA01 (50 µmol/L; Bio-Techne) was used to inhibit CaCC activity.

Statistical analysis

Data are presented as means ± standard deviation. Differences between means were statistically analyzed by ANOVA, using the Dunnett correction to control for multiple comparisons (SPSS 28, IBM).

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Chapter 9

Summary and General discussion

Summary

The work presented in this thesis focuses on bicarbonate transport mediated by the CFTR channel in primary cell cultures, with a specific interest in its role in the etiology of pancreatitis and its improvement by CFTR modulators. The first part centers on CFTR-mediated anion transport in subjects carrying *CFTR* variants, whereas the second part centers on the development of models for studying pancreatic CFTR function.

In **chapter 2**, we reviewed the current knowledge of CFTR-dependent bicarbonate transport in pancreatic ductal cells, and its role in the development of CF, and CFTR-related and acquired forms of pancreatitis.

The production of alkaline pancreatic juice is strongly dependent on CFTR, which is activated upon postprandial stimulation of ductal cells by secretin. This hormone triggers cyclic AMP (cAMP) production, and protein kinase-mediated phosphorylation and activation of CFTR. Through the coordinate action of CFTR and other ion transport mechanisms, the bicarbonate concentration in human pancreatic juice can reach levels of up to 140 mmol/L. These base equivalents serve to neutralize gastric acid entering the duodenum, and aid nutrient digestion.

In view of the important role of CFTR in the production of alkaline pancreatic juice, it is perhaps not surprising that when loss of CFTR function occurs, as in cystic fibrosis (CF), exocrine pancreatic function is severely compromised. As in other hereditary forms of pancreatitis, environmental factors seem to have little influence on disease progression in this scenario. Conversely, for those carrying *CFTR* variants that permit residual channel function and do not cause CF, environmental factors are increasingly important in determining disease liability. In fact, pancreatitis in this cohort is often triggered by an external stressor, e.g. the ingestion of a fatty meal or alcohol. The high incidence of *CFTR* mutations observed in the pancreatitis population, indicates that the exocrine pancreas is highly vulnerable to even small perturbations in ductal CFTR activity. Initially silent mutations, although not causing overt CF-like pathology, may sensitize the pancreas to a second hit. Such *CFTR* alleles may only have a small effect on the individual risk, but on the population level they may significantly increase the risk of developing pancreatitis.

Part I Pancreatitis in carriers of *CFTR* variants

None of the bioassays currently used for diagnostic purposes can accurately assess CFTR-mediated bicarbonate transport. Therefore, because of the particular relevance of CFTR-dependent bicarbonate transport in the pancreas, and because *CFTR* mutations may reduce the bicarbonate permeability of CFTR, for the work contained in this thesis, we employed assays that were designed to specifically assess CFTR-mediated

bicarbonate transport in patient tissues. In **chapters 3-5**, we used these assays to screen a range of (mostly) rare *CFTR* mutations, and also tested the effect of modulators on CFTR-mediated bicarbonate vs. chloride transport.

Among various other CFTR variants evaluated, this analysis included four carriers of the D1152H allele, one of the purported CFTR-BD variants (**chapter 4**). These four subjects carried this variant *in trans* of a minimal function allele, i.e. a class I mutation. While the levels of residual CFTR-mediated chloride and bicarbonate transport between these different subjects with similar *CFTR* genotype varied, in all the D1152H lines assayed, we observed that chloride transport was lowered compared to wild type controls. Moreover, in all, modulator treatment, apart from enhancing bicarbonate transport, also markedly improved chloride transport function. Collectively, this argues against the contention that the D1152H mutation confers a specific defect in bicarbonate permeability of CFTR. In fact, we found that none of the variants investigated in **chapters 3-5** demonstrated a specific defect in bicarbonate transport, and thus were unable to confirm their existence.

Our studies included an analysis of CFTR activity in a cohort of pancreatitis patients carrying mutations in *CFTR* (**chapter 5**). It showed that CFTR dysfunction in pancreatitis patients carrying *CFTR* variants is relatively prevalent, but no specific defect in CFTR-mediated bicarbonate transport was detected. Importantly, some subjects in this cohort with a lowered CFTR activity carried a wild type CFTR allele, suggesting that disease-causing mutations may also be located outside the *CFTR* locus. The study showed that patient-derived organoids may be used to assess the impact of *CFTR* genotype on epithelial bicarbonate and chloride transport, and the response to CFTR modulator therapy.

Part II Pancreatic ductal models to study CFTR function

The function of CFTR in human pancreatic ducts of people with CF has not been assessed, mainly because of the current lack of suitable *in vitro* or *in vivo* bioassays (1). Therefore, in the second part of this thesis, we explored the use of pancreatic tissue to culture ductal organoids and perform CFTR assays.

To model the pancreatic ductal tree during pancreatitis, we prepared organoids from porcine pancreas, and used these to assess the effect of inflammation on ductal CFTR-dependent anion transport (**chapter 6**). Our results indicate that select cytokines that are key mediators of the pancreatic inflammatory response markedly increased expression of CFTR in ductal epithelium, and stimulated CFTR-mediated ductal anion secretion. As was observed for other epithelia (e.g. airways), CFTR induction required a combination of pro-inflammatory cytokines (2). Stimulation of CFTR activity, by enhancing the secretory capacity of the ductal epithelium, may mitigate the effects of external (metabolic) stresses located within the ducts, attenuating or preventing

further tissue injury and containing the inflammatory response in the initial stages of a pancreatitis event. These results point to a more central role of the ductal compartment, and CFTR in particular, in the etiology of pancreatitis.

Pancreatitis may not only be linked to CFTR mutations, but may also result from environmental factors that reduce (wild type) CFTR function. We exploited the organoid model to test the hypothesis that ethanol and/or unsaturated fatty acids affect ductal CFTR function (**chapter 7**). We found that ethanol and unsaturated fatty acids had only marginal effects on ductal cell viability, and even prolonged exposure did not attenuate ductal, CFTR-mediated anion secretion, which argues against a direct involvement of acquired CFTR dysfunction in the etiology of alcohol-related pancreatitis.

To assess pancreatic CFTR function in patients with pancreatitis, we set out to establish ductal organoids from patient material (**chapter 8**). Tissue was collected by per-oral pancreatoscopy, from the main pancreatic duct using a biopsy forceps, when subjects diagnosed with calcifying chronic pancreatitis underwent electrohydraulic lithotripsy. We found that such tissue samples allowed the culture of ductal epithelial cells. Importantly, these cultures displayed CFTR-dependent anion and fluid transport, and we found that CFTR modulators improved ductal anion and fluid secretion in one of these cases. Therefore, we propose that this model may be exploited for drug testing, ultimately to improve treatment of CP.

General discussion and future perspectives

CFTR as a bicarbonate channel

In the pancreas, CFTR is particularly important for attaining the exceptionally high concentration of bicarbonate observed in pancreatic juice. We found that CFTR in pancreatic ductal cells chiefly mediates secretion of bicarbonate, rather than chloride (**chapter 6**), similar to human primary cholangiocytes (3) and guinea pig pancreatic duct cells (4), but unlike intestinal and airway epithelium (5-8). Importantly, CFTR-dependent bicarbonate secretion was maintained in the presence of an unfavorable electrochemical gradient, indicating CFTR may be crucial for attaining the high bicarbonate concentration of pancreatic juice observed in the distal duct (4, 9). The human pancreatic ductal organoids (hPDOs) used in our studies, displayed CFTR-mediated currents of up to 80 $\mu A/cm^2$, which was higher than reported in a previous study (30 $\mu A/cm^2$) (10). This may reflect differences in CFTR activity along the pancreatic ductal tree, and/or the method of tissue collection. As pancreatic organoids are still a relatively novel model, further studies are needed to elucidate functional differences between the separate segments of the ductal tree. Of relevance to ductal physiology, we also demonstrated the presence of calcium activated chloride channels (CaCCs) in porcine and human PDOs, in line with other recent observations (11, 12). Together, these observations help uncover the mechanisms underlying ductal anion secretion and underline the cell type specific anion transport properties of CFTR.

It has been proposed that a select group of CFTR- missense mutations specifically reduce the permeability of the CFTR channel pore to bicarbonate, and confer an increased risk of developing pancreatitis (13). Such so-called bicarbonate defective (CFTR-BD) mutations may also interfere with the regulation of the anion permeability of CFTR by WNK-type protein kinases. From our work it appears that the link made between the reduced bicarbonate transport and some of the purported CFTR-BD variants, and in extension the risk of pancreatitis, is tenuous (**chapters 4 and 5**). This is also indicated by more recent studies, which refuted that one of the CFTR-BD variants, R75Q, is a risk factor for CP (14). Similarly, D1152H does not seem to be strongly linked to pancreatic dysfunction, as pancreatic insufficiency is less prevalent in D1152H-homozygote CF patients than the general CF population (15). Thus, epidemiological evidence that CFTR-BD variants preferentially affect organs that mediate bicarbonate transport varies (4, 13, 14, 16-18).

An important limitation of our studies in part I of this thesis is that we used intestinal cells for our assays, whereas purported defects in CFTR-dependent bicarbonate transport may only be apparent in tissues which are particularly affected (e.g. pancreas, or the vas deferens of the male reproductive tract). The use of a “surrogate” intestinal model was mainly instigated by the fact that, at present, pancreatic tissue cannot

routinely be sampled from patients and no validated method is available that allows direct assessment of CFTR function in ductal epithelium. However, the possibility to use pancreatic ductal, patient-derived tissue to assess CFTR function in the context of pancreatitis is explored in part II of this thesis. A further limitation of electrophysiological studies on intact cells is that they cannot be used to assess the relative permeability of CFTR variants to anions. Nevertheless, even though only transepithelial CFTR-dependent chloride and bicarbonate transport was assessed, it is of interest that in several CFTR variants (like L1065P and R1066H), modulator treatment appeared to restore bicarbonate transport more effectively than chloride transport (**chapter 3**). Similar was observed in some patients carrying D1152H-CFTR, which indicates that the response to modulators may vary between patients carrying the same variant (**chapter 4**). Previously, CFTR modulators have been shown to change the relative permeability of CFTR to anions (19, 20), and rescue of bicarbonate secretion may differ between tissues (3). This implies that beneficial effects of CFTR modulators observed in other cell types are not necessarily reproduced in the pancreatic ductal epithelium.

Pancreatitis as a CFTR-RD

Diagnosing CFTR-RD

Because of the association with *CFTR* variants, pancreatitis may be considered a typical CFTR-related disorder (CFTR-RD). Whereas classically, CFTR-RD was defined as a single organ disease with evidence of CFTR dysfunction in the absence of CF diagnosis, recently updated guidelines do not exclude a diagnosis of CFTR-RD when more than one organ is affected. The diagnosis of CFTR-RD requires evidence of CFTR dysfunction (*in vivo* or *ex vivo*, in at least two different functional tests) and/or the presence of *CFTR* variant(s) known to reduce CFTR function (with at most one CF-causing variant) (21). Clear guidelines and increased awareness among specialists will help to minimize under-diagnosis of those with non-specific symptomatology (22). However, several diagnostic challenges remain to be overcome, e.g. genetic testing is costly and not routinely recommended for single organ CFTR-RD, appropriate CFTR bioassays are unavailable at most institutions, and subjects may not be referred to CF centers (21).

CFTR variants

Unlike in CF, the *CFTR* genotype as a surrogate marker for CFTR-RD diagnosis is challenging, because CFTR-RD is commonly associated with the presence of at least one *CFTR* allele with undefined clinical and/or functional consequences. *CFTR* alleles found in CFTR-RD may be classified as non-CF causing variants, variants of varying clinical consequence (VVCC), or variants of uncertain significance (VUS). The application of non-mutation specific genetic analysis, e.g. exon sequencing, for diagnosis of CFTR-RD and CFSPID/CRMS has led to the identification of a large number of VUS (22), Extensive

epidemiological studies are indicated to assess the penetrance of these variants. Databases such as CFTR-France may help to elucidate the genotype-phenotype relation of VUS (23, 24). The inclusion of clinical information on the disease manifestations in separate organs in carriers of *CFTR* variants, can help to estimate the disease liability not just for CF, but also for CFTR-RD.

CFTR bioassays

Because the complex relationship between genotype and phenotype often limits the clinical utility of genetic information, there is a need for additional *in vitro* characterization of the impact of separate *CFTR* variants on channel function (22, 25). Whereas severe mutations lead to the absence or near absence of CFTR function (< 10% of normal), typical of CF, CFTR function is generally thought to be between 10-50% of normal in CFTR-RD (22). Such more subtle disease-causing defects may be more challenging to assess in bioassays that were designed for diagnosis of CF. Results of different bioassays may be inconsistent, because CFTR activity varies between tissues, and because tests assess different functions of CFTR. While these tests may discriminate CF from non-CF, their potential for delineating partial levels of CFTR function and thus discerning CFTR-RD from CF and unaffected individuals is limited (26).

In **chapter 5**, we observed that in non-CF pancreatitis patients, Gibson-Cooke sweat test (GCST), bubble sweat test (BST), nasal potential difference (NPD) and intestinal short-circuit current measurement (ICM), often yielded inconsistent results. These observations align with previous studies showing that in patients carrying VVCCs, either *in trans* of a CF-causing or a WT allele, CFTR function, as assessed in established bioassays, may vary considerably at different points in time and between tissues (27, 28). This implies that bioassays performed on other tissues than the site of disease (e.g. NPD, GCST, ICM for pancreatitis patients) must be interpreted with caution. Consequently, it is crucial that multiple diagnostic procedures are used to evaluate CFTR function in pancreatitis patients carrying *CFTR* variants.

Abnormal biomarkers of CFTR function have also been found in subjects carrying a *CFTR* variant on a single allele (e.g. **chapter 5**) (28-33). The development of CFTR-RD in *CFTR* heterozygotes points towards a multifactorial etiology, which includes non-*CFTR* genetic- and environmental factors (22). Indeed, more than a third of the subjects investigated in **chapter 5** carried mutations in at least one of the other genes involved in pancreatitis, supporting a multifactorial mechanism.

Therapy for CFTR-RD

It is challenging to distinguish CFTR-RD from mild CF (26) (see Angyal et al. Pancreatology 2024, Letter to the editor (34) and reply (35)), and it seems reasonable to assume that patients with CFTR-RD (with poly-organ manifestation) may benefit

from modulator therapy (22). In our studies, IVA improved CFTR-dependent bicarbonate transport in a small group of pancreatitis patients, suggesting modulator therapy could counter luminal acidification and decrease mucus viscosity in pancreatic ductal epithelium (2,3). However, in CFTR-RD, i.e. in the presence of residual CFTR function, the clinical benefit of a certain improvement in CFTR function is less clear than in CF patients without residual CFTR function (36). Therefore, further evaluation is warranted.

Chronic pancreatitis

Chronic pancreatitis (CP) is a complex, multifactorial disease and CFTR dysfunction is likely one of the contributing factors (37). The TIGAR-O classification is a comprehensive checklist to assess all factors contributing to pancreatitis, including *CFTR* mutations and environmental/metabolic factors such as alcohol consumption, cigarette smoking and bile acid exposure, that may affect CFTR function (38). Current guidelines recommend genetic testing in patients younger than 20 years and/or those with a family history of two or more first- or second-degree relatives with CP (39). In adults with idiopathic CP and no further clinical signs of CF, only a chloride iontophoresis should be performed to rule out CF (39). In **chapter 5**, four adults with pancreatitis were (provisionally) diagnosed with CF, all with (slightly) elevated GCST levels, underlining the importance of performing this test in adults. However, as previously discussed, this biomarker has limited diagnostic accuracy. Probably, many CFTR-associated pancreatitis cases remain unidentified. It has been estimated that up to 30% of those with idiopathic pancreatitis carry mutations in the *CFTR* gene (40, 41). Identifying pathogenic *CFTR* variants early, preferably after an initial attack of AP, may be of clinical benefit, as it provides a window of opportunity for CP-preventing interventions, when they become available (42).

Beneficial effects of CFTR modulators have been observed in animal models of pancreatitis (43-45), in hPDOs *in vitro* (12) and CF patients (46-50). These studies suggest that rescue of CFTR function by modulators can ameliorate the severity of (subsequent) pancreatitis episodes (44), preventing progression of tissue damage in the continuum of AP to RAP and CP. (43). In our study, we obtained ductal tissue from CP patients, that, at this stage, is thought to have undergone extensive tissue remodeling. Our findings, i.e. subtle improvement in CFTR function in CP hPDOs are promising, suggesting that even in this disease stage CFTR function can partially be restored. Clinical relevance at different stages of disease remains to be evaluated, especially as pancreatic insufficiency might transition into sufficiency with increased risk of pancreatitis (51).

Concluding remarks

CFTR dysfunction is involved in the pathology of both genetic and acquired forms of pancreatitis. In this thesis, we point out the challenges related to identifying patients with CFTR-related pancreatitis, based on currently, routinely applied biomarkers. Moreover, we investigated the potential and limitations of novel bioassays on patient-derived tissue and primary cell cultures, to improve diagnosis of (CFTR-related) pancreatitis. We show that pancreatic organoids generated from per-oral biopsy can be employed to assess potential beneficial effects of CFTR-modulator therapy. We propose that in the future, therapies aimed at restoring or enhancing CFTR activity may be considered to treat pancreatitis and that these techniques may enable adequate selection of patients.

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Appendices

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Nederlandse samenvatting

Dit proefschrift beschrijft onderzoek naar de rol van bicarbonaattransport gemedieerd door het CFTR-kanaal in het ontstaan van alvleesklierontstekingen de verbetering daarvan door CFTR-modulatoren. Het eerste deel van dit werk concentreert zich op CFTR-gemedieerd aniontransport bij personen die een *CFTR* gen-variant dragen. Het tweede deel richt zich op de ontwikkeling van modellen voor het bestuderen van CFTR-functie in de alvleesklier.

In **hoofdstuk 2** heb ik de huidige kennis over CFTR-afhankelijk bicarbonaattransport in alvleesklierbuis cellen uiteengezet, evenals de rol van dit transport in het ontstaan van taaislijmziekte (ook wel cystische fibrose (CF) genoemd), en van CFTR-gerelateerde en verworven vormen van alvleesklierontsteking.

De productie van alkalisch alvleesklier sap is sterk afhankelijk van CFTR. CFTR wordt geactiveerd na een maaltijd na stimulatie van cellen in de alvleesklierbuis door secretine. Dit hormoon induceert de productie van cyclisch AMP (cAMP) en kinase-gemedieerde fosforylering en activatie van CFTR. Door de gecoördineerd ion transport, gemedieerd door onder andere CFTR, kan de bicarbonaatconcentratie in humaan alvleesklier sap oplopen tot 140 mmol/L. Deze base-equivalenten neutraliseren het maagzuur dat de twaalfvingerige darm binnenkomt en ondersteunen de vertering van voedingsstoffen.

Gezien de belangrijke rol van CFTR in de productie van alkalisch alvleesklier sap, is het niet verrassend dat verlies van CFTR-functie, zoals bij CF, leidt tot ernstige aantasting van de exocriene alvleesklier functie. Net als bij andere erfelijke vormen van alvleesklierontsteking, lijken omgevingsfactoren in dit scenario weinig invloed te hebben op het ziekteverloop. Daarentegen wordt bij dragers van *CFTR*-varianten, die restfunctie van het kanaal behouden en geen CF veroorzaken, steeds duidelijker hoe belangrijk omgevingsfactoren zijn in het bepalen van de vatbaarheid voor ziekte. In deze groep wordt alvleesklierontsteking vaak uitgelokt door een externe prikkel, zoals de inname van een vetrijke maaltijd of alcohol. De hoge incidentie van *CFTR*-mutaties in de populatie met alvleesklierontsteking populatie wijst erop dat de exocriene alvleesklier zeer kwetsbaar is voor zelfs kleine verstoringen in de alvleesklier buis CFTR-activiteit. Aanvankelijk stille mutaties veroorzaken mogelijk geen duidelijke CF-achtige pathologie, maar kunnen de alvleesklier wel gevoeliger maken voor een tweede schadelijke prikkel. Dergelijke *CFTR*-allelen hebben mogelijk slechts een klein effect op het individuele risico, maar kunnen op populatieniveau het risico op alvleesklierontsteking aanzienlijk verhogen.

Deel I Alvleesklierontsteking bij dragers van CFTR-varianten

Geen van de bioassays die momenteel voor diagnostische doeleinden worden gebruikt, kan CFTR-gemedieerd bicarbonaattransport nauwkeurig beoordelen. Daarom hebben wij, vanwege de bijzondere relevantie van CFTR-afhankelijk bicarbonaattransport in de alvleesklier en omdat *CFTR*-mutaties de bicarbonaatdoorlaatbaarheid van CFTR kunnen verminderen, voor het werk in dit proefschrift assays gebruikt die specifiek ontworpen zijn om CFTR-gemedieerd bicarbonaattransport in patiëntweefsels te meten. In **hoofdstukken 3–5** gebruikten we deze assays om een reeks (voornamelijk) zeldzame *CFTR*-mutaties te screenen en testten we tevens het effect van modulatoren op CFTR-gemedieerd bicarbonaat- versus chloridetransport.

Onder de verschillende onderzochte CFTR-varianten bevonden zich vier dragers van het D1152H-allel, een van de vermeende CFTR-BD-varianten (**hoofdstuk 4**). Deze vier personen droegen deze variant *in trans* met een minimale-functie-allel, oftewel een klasse I-mutatie. Hoewel de niveaus van resterend CFTR-gemedieerd chloride- en bicarbonaattransport tussen deze personen met een vergelijkbaar *CFTR*-genotype varieerden, zagen we in alle D1152H-lijnen een verlaagd chloridetransport ten opzichte van wildtype-controles. Bovendien verbeterde behandeling met modulatoren in alle gevallen niet alleen het bicarbonaattransport, maar ook duidelijk de chloride-transportfunctie. Gezamenlijk pleiten deze bevindingen tegen de veronderstelling dat de D1152H-mutatie een specifiek defect veroorzaakt in de bicarbonaatpermeabiliteit van CFTR. Sterker nog, geen van de varianten onderzocht in **hoofdstukken 3–5** vertoonde een specifiek defect in bicarbonaattransport, waardoor wij het bestaan daarvan niet konden bevestigen.

Onze studies omvatten ook een analyse van CFTR-activiteit in een cohort van patiënten met alvleesklierontsteking met *CFTR*-mutaties (**hoofdstuk 5**). Hieruit bleek dat CFTR-disfunctie relatief frequent voorkomt bij patiënten met alvleesklierontsteking met *CFTR*-varianten, maar dat geen specifiek defect in CFTR-gemedieerd bicarbonaattransport werd aangetoond. Belangrijk is dat sommige personen in dit cohort met verlaagde CFTR-activiteit een wildtype CFTR-allel droegen, wat suggereert dat ziekte veroorzakende mutaties ook buiten de *CFTR*-locus kunnen liggen. De studie liet zien dat patiënt-afgeleide organoïden gebruikt kunnen worden om de invloed van het *CFTR*-genotype op epitheliaal bicarbonaat- en chloridetransport en de respons op CFTR-modulatortherapie te beoordelen.

Deel II Alvleesklierbuis modellen voor het bestuderen van CFTR-functie

De functie van CFTR in humane alvleesklierbuizen van mensen met CF is nauwelijks onderzocht, voornamelijk door een gebrek aan geschikte *in vitro*- of *in vivo*-bioassays. Daarom hebben we in het tweede deel van dit proefschrift o.a. een organoïd

modelsysteem ontworpen op basis van weefsel gewonnen uit de alvleesklierbuis voor het uitvoeren van CFTR-assays.

Om de buis tijdens alvleesklierontsteking te modelleren, maakten wij eerst organoïden van varkensalvleesklier en gebruikten deze om het effect van ontsteking op CFTR-afhankelijk aniontransport in de buizen te beoordelen (**hoofdstuk 6**). Onze resultaten wijzen erop dat bepaalde cytokinen, belangrijke mediatoren van de alvleesklier ontstekingsrespons, de expressie van CFTR in buis epitheel sterk verhoogden en CFTR-gemedieerde buis anionsecretie stimuleerden. Zoals ook bij andere epithelia (bijvoorbeeld de luchtwegen) is waargenomen, vereiste inductie van CFTR een combinatie van pro-inflammatoire cytokinen. Stimulatie van CFTR-activiteit kan, door de secretoire capaciteit van het buis epitheel te vergroten, de effecten van externe (metabole) stressfactoren in de buizen verminderen, verdere weefselschade beperken en de ontstekingsrespons in de vroege fasen van alvleesklierontsteking afremmen of voorkomen. Deze resultaten wijzen op een centralere rol van het alvleesklierbuis compartiment, en in het bijzonder van CFTR, in de etiologie van alvleesklierontsteking.

Alvleesklierontsteking kan niet alleen samenhangen met *CFTR*-mutaties, maar ook het gevolg zijn van omgevingsfactoren die de functie van (wildtype) CFTR verminderen. Wij gebruikten het organoïd model om de hypothese te testen dat ethanol en/of onverzadigde vetzuren invloed hebben op CFTR-functie in de buis (**hoofdstuk 7**). We vonden dat ethanol en onverzadigde vetzuren slechts marginale effecten hadden op de levensvatbaarheid van buiscellen, en dat zelfs langdurige blootstelling CFTR-gemedieerde anionsecretie niet verminderde. Dit pleit tegen een directe rol voor verworven CFTR-disfunctie in de etiologie van alcohol gerelateerde alvleesklierontsteking.

Om CFTR-functie in de alvleesklier bij patiënten met alvleesklierontsteking te beoordelen, hebben we een methode ontworpen om buis organoïden te verkrijgen uit patiëntmateriaal (**hoofdstuk 8**). Weefsel werd verzameld via per-orale pancreatoscopie uit de hoofd alvleesklierbuis met behulp van een biopsietang, wanneer patiënten met calcificerende chronische alvleesklierontsteking elektrohydraulische steenvergruizing ondergingen. Wij vonden dat dergelijke weefselmonsters geschikt waren voor de kweek van buis epitheelcellen. Belangrijk is dat deze organoïd modellen CFTR-afhankelijk anion- en vloeistoftransport vertoonden, en dat CFTR-modulatoren in één van deze gevallen de buis anion- en vloeistofsecretie verbeterden. Daarom stellen wij voor dat dit model gebruikt kan worden voor medicatietesten, met als uiteindelijk doel de behandeling van chronische alvleesklierontsteking te verbeteren.

PhD portfolio

Description	Organizer	EC
UEG Week (2020)	UEG	1.20
Organoid training (2021)	HUB	0.25
Erasmus MC PhD day 2021 (2021)	Erasmus MC Graduate School	0.20
Applications of Organoid Technology Symposium (2021)	MDI Biological Laboratory	0.80
European Pancreatic Club Meeting (2021)	European Pancreatic Club	0.85
HIT CF 2.0 (2021)	NCFS	0.25
Scientific Integrity (2021)	Erasmus MC Graduate School	0.30
Photoshop and Illustrator CC (2021)	Erasmus MC Graduate School	0.30
Master student supervision (2021)		7.50
UEG Week (2021)	UEG	1.20
UEG Postgraduate teaching (2021)	UEG	0.40
Erasmus MC - SNP Course: SNPs and Human Diseases (2021)	The Erasmus Postgraduate School Molecular Medicine	2.00
Biosafety – working in ML-I and ML-II laboratories (2021)	Erasmus MC Academie	0.20
Biomedical Sciences PhD day 2022 (2022)	Erasmus MC Graduate School	0.30
European Cystic Fibrosis Conference (2022)	ECFS	1.00
European Pancreatic Club Meeting (2022)	European Pancreatic Club	1.10
UEG Week (2022)	UEG	1.50
Bachelor student supervision (2022)		10.80
HPSG Meeting (2022)	Hungarian Pancreatic Study Group	0.50

Curriculum vitae



Dóra Angyal was born on the 1st of August 1995 in Budapest, Hungary. In 2004, she moved to Eindhoven, the Netherlands. After completing her Dutch high school education in 2013, she began her medical studies at the Erasmus Medical Center in Rotterdam. During her undergraduate years, she worked at the Lung Analysis research group. Alongside her medical training, she pursued two other master degrees, one in health care management and another in molecular medicine, reflecting her interest in the broader healthcare landscape and the mechanisms underlying human disease. She graduated in 2020.

Her interest in translational medicine and the gastrointestinal system led her to pursue a PhD in the department of Prof.dr. Marco Bruno and the laboratory of Prof.dr. Maikel Peppelenbosch at the Erasmus MC. Her doctoral research focused on CFTR-mediated ion and fluid transport across epithelial cells of the intestine and pancreas, under supervision of dr. Hugo de Jonge and dr. Marcel Bijvelds. She investigated personalized medicine approaches and established novel disease models to study CFTR-related pancreatitis. In 2023, Dora transitioned into clinical practice at the Reinier de Graaf Hospital in Delft, initially in the department of Internal Medicine and later in Gastroenterology. She continued her clinical work in the department of Gastroenterology at the Erasmus MC and UMC Utrecht. Currently, Dora is a resident Gastroenterology at the UMC Utrecht.

Dankwoord

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Lieve **Ferdy** en **Lina,** wij waren het dreamteam. Onze vriendschap, die ontstond tijdens de eerste stappen als arts, bevestigde dat ik met mensen zoals jullie in de kliniek wil blijven werken. Jullie waren een mooie motivatie om toch die weekenden aan mijn promotie te besteden, en hoewel mijn boekje niet zo dik is als die van jullie, mag het resultaat er zeker zijn.

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Lieve **Lieke, Timon** en **Sjoerd**. Als we elkaar zien, is er één ding zeker: er is altijd lekker eten. Ik begin steeds meer te geloven dat vriendschap ook door de maag kan gaan. Ook al zien we elkaar niet zo vaak, het is altijd fijn om even een frisse blik te krijgen op hoe het leven loopt. En om eraan herinnerd te worden dat we niet alles zo serieus moeten nemen (in ieder geval niet de PhD).

Dear **Irina** and **Kiki**, my oldest friends, who have known me through a lot of phases, moving away to different big cities, and now coming together in Utrecht. When we meet up, it feels like nothing has changed, even though we have all grown in different ways. I

am thankful that you have stood by me all these years and look forward to seeing you more often! Dearest Kiki, thank you so much for the artwork you created for this thesis. You are incredibly talented and your design truly brings my thesis to life.

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Lieve Club4, lieve **Jorien, Roos, Jet** – met een kop thee kunnen we alle problemen in de wereld oplossen, en anders is er nog wijn. Ons twee jaar roeien heeft een band geschept waarvan ik hoop dat die voor het leven is. Met jullie kon ik sparren over wat belangrijk is in het leven, waar je wel en niet invloed op hebt, en hoe daarmee om te gaan. Met jullie kan ik me jong voelen en uitgaan in Oslo, maar ook een oma voelen en puzzelen in een huisje in de Zwitserse bergen. Ik hoop dat we nog vele avonturen samen beleven.

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most vagyok. Köszönöm, hogy bíztatok bennem, ott voltatok, és most is számíthatok rátok minden helyzetben.

Dear **Levi**, today we not only celebrate the completion of my PhD research, but also that we've been together for 12 years. I would like to thank you for your endless love and unwavering support. You were always there to listen when I needed to vent, patiently enduring my presentations over and over, even when they dragged on way past bedtime, and gave me a push when I needed it. You've celebrated every success with me, no matter how small, and reminded me why what I do matters. Your (often acts, not words of) kindness, patience, and belief in me have made this journey so much easier. I am truly grateful to have you by my side. Ons leven samen is niet saai. Hierna wordt het rustiger… of misschien toch niet.

