Novel diagnostic and therapeutic opportunities for Cystic Fibrosis

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Novel diagnostic and therapeutic opportunities for Cystic Fibrosis

Nieuwe diagnostische en therapeutische mogelijkheden voor cystic fibrosis

(met een samenvatting in het Nederlands)

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General introduction

1

Cystic fibrosis (CF) is the most common life-shortening rare disease caused by mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene, which was identified in 1989 [1-3]. The *CFTR* gene encodes a 3',5'-cyclic adenosine monophosphate (cAMP)-activated transmembrane anion channel and mutations herein disturb ion transport at the apical membrane, mainly of mucosal surfaces. CF is a multi-organ disease due to widespread expression of the CFTR protein in the upper and lower airways, pancreas, bile ducts, gastrointestinal tract (GI), vas deferens, sweat glands, some immune cells, and other tissues. Patients experience many different symptoms such as intestinal obstruction, malnutrition, infertility, salty sweat, but most ultimately succumb to recurrent lung infections and associated inflammation and tissue damage [4]. Currently, the median life expectancy for newborn CF-patients, with the most common disease causing mutation, was estimated in 2010 at 37 years [5].

The main CF research challenge is to find novel therapies that can lead to a real cure for all patients. New developments have shown that remarkable treatment effects can be observed upon treatment of the mutant CFTR protein by pharmacotherapy. These therapies are currently insufficient to 'cure' CF for the majority of patients, and act variably between individuals. New drug discovery and drug validation platforms that enable the effective translation of *in vitro* data to clinical effects are therefore highly needed. Furthermore, models that help the identification of drug responsive patients as well as monitoring tools to evaluate the *in vivo* efficacy may further assist drug development, and a more personalized application. I first describe the different disease manifestations of CF, and how these relate to the molecular basis of disease. An update on the currents status of drug development for CF is provided, particularly emphasizing on drugs that can modulate CFTR function. Finally, I indicate how work from this thesis is aiming to develop novel methodology for a more effective identification and application of CFTR modulating drugs.

Cystic Fibrosis

In many European countries the name for cystic fibrosis is associated with the observed thickened mucus, for example in Dutch, French and German the names are 'taaislijmziekte', 'mucoviscidose' and 'Mukoviszidose', respectively. Indeed, viscous mucus appears the general mechanism underlying the disease, as accumulation of thick viscous mucus leads to obstruction of exocrine ducts in many different organs resulting in various organ-specific disease manifestations.

The respiratory tract

The respiratory tract is severely affected in CF, and a progressive decline in lung function of approximately 1-2 % per year is the main cause leading to CF related early death around the third or fourth decade of life [6]. Loss of pulmonary function (as monitored by forced expiratory volume in 1 second (FEV_1)) is associated with chronic infections with opportunistic bacteria such as *S. aureus* and *P. aeruginosa*, and characterized by obstruction of the lower airways by mucus and extensive bronchiectasis [7-10]. Most patients eventually require lung transplantation that is associated with a median actuarial survival time of approximately 7.5 years [11].

Abnormalities in electrophysiological properties of the upper airways are used as a

diagnostic marker for difficult-to-diagnose CF [12]. Loss of CFTR function is associated with hyper absorption of sodium due to upregulation of the epithelial sodium channel (ENaC), and defective (β-adrenergic induced) chloride secretion [13]. Clinical features observed in the upper airways of CF patients include frequent nasal polyps, sinusitis and colonization with pathogens. CF nasal polyps are different from those observed in for example asthma, as they lack dense eosinophilic infiltrates and are highly enriched with mucins [14-15]. It has been suggested that the upper respiratory tract, including the sinuses, may be a reservoir for adapted opportunistic pathogens that may "drip" into the lower respiratory tract [16], thereby contributing to chronic infection and survival of bacteria despite heavy use of inhalation antibiotics.

New data indicates that already soon after birth the upper and lower CF airways display altered innate defense mechanisms and sticky mucus that subsequently drives the acquisition of a distinctive microbiome and an aberrant inflammatory phenotype [17]. The distinct microbiome development in infants with CF is in part caused by antibiotic usage [18]. In newborn CF piglets, impaired detachment of thickened and sticky mucus and air-trapping was already observed directly after birth [19-20]. Data furthermore indicate an important role for CFTR-mediated bicarbonate secretion next to chloride [21]. Lower bicarbonate transports results in reduced chelation of calcium from tightly packed mucins upon their secretion by goblet cells, resulting in improper unfolding and hydration of mucins [22-26]. The resulting sticky mucus may directly contribute to a limited 'sterile' inflammatory responses in epithelial cells, albeit that this may be species-specific [27]. In mice and ferret "sterile" inflammation was observed, whereas in pigs infection precedes inflammation [28-29]. Furthermore, the defective bicarbonate secretion acidifies the airway surface liquid (ASL) that reduces the activity of antimicrobial peptides in the ASL [30-31]. CF mice acquire a clear CF pulmonary phenotype upon introduction of a proton pump that is expressed in pigs and humans, further indicating the important of pH for the establishment of a CF pulmonary phenotype [32]. Finally, the acidified ASL contributes to impaired neutrophil and macrophage function [33-34] and reduced mucociliary beat frequency [35]. These studies indicate that various innate defense defects present at birth contribute to the prolonged exposure to pathogens and inflammation characteristic for CF [36] (Fig. 1), and urge the development of early interventions to limit chronic infections and irreversible tissue damage.

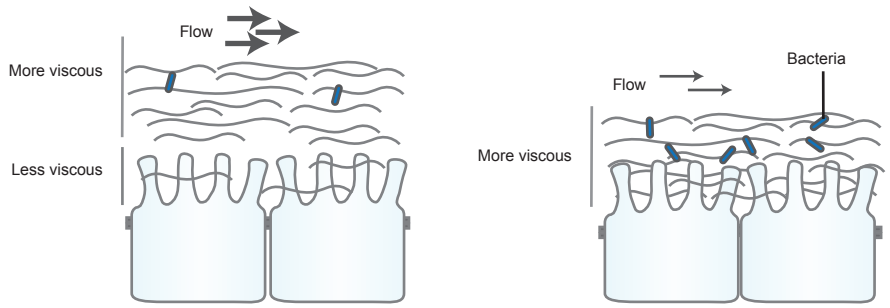

Figure 1. CFTR function in airways. Absence of CFTR induces thickened mucus resulting in a disruption of the biphasic mucus layer, preventing mucociliary clearance of exogenous material such as bacteria.

The pancreas

Fibrosis of the pancreas in one of the hallmarks of severe CF. In 1595 the Dutch professor Pieter Pauw at Botany and Anatomy, conducted an autopsy on an 11 year old girl who possibly died of CF and noted that the pancreas was white, swollen and enriched with mucus [37-38] (Fig. 2). The exocrine function of the pancreas is mostly affected in CF, resulting in a decreased release of digestive enzymes and bicarbonate (to neutralize the low pH of the stomach content) into the duodenum [39-40] (Fig. 3). The important role for CFTR-dependent bicarbonate transport in the pancreas is exemplified by *CFTR* mutations that are permeable for chloride but relatively impermeable for bicarbonate. Mutations associated with impermeability for bicarbonate are also associated with pancreas insufficiency [21]. The impaired CFTR function in the pancreas results in mucus plugging of the exocrine ducts and accumulation of digestive enzymes in the ducts is likely causing pancreas exocrine dysfunction. Also the pancreas endocrine function can be impaired in CF patients. Especially since patients become older, the destruction of pancreatic β cells results in insufficient insulin production and subsequent CF-related diabetes that affects approximately 25% of patients beyond 20 of age [41].

Figure 2. Pieter Pauw (1564-1617) a Dutch professor at botany and anatomy documented the swollen pancreas of a 11 year old girl who possibly died of CF in 1595 (Picture obtained from: Wikipedia.org).

Pancreatic stress-related markers in blood such as immune reactive trypsinogen (IRT) and pancreatitis-associated protein (PAP) are currently used for newborn screening of CF, which was initiated in 2011 in the Netherlands [42]. The progression of pancreatic disease is variable between patients, but most patients with severe CF show a clear loss of exocrine pancreas function and require pancreatic enzyme replacement treatment starting in the first years of life. In contrast, many patients with CF that have a milder disease course retain sufficient pancreas function. Pancreatic-(in)sufficiency is commonly used to type the clinical severity of CF disease.

The disturbed exocrine pancreas function is currently treated with enzyme replacement therapy (ERT) and pH neutralizing agents [43]. These therapies were already developed in the early 50's and ensured that CF patients would survive their first decade of life. It was commonly believed that pancreas insufficiency is irreversible, but the use of novel CFTR-modulating drugs appears to indicate that pancreatic function may be restored to some extent [44]. This is indicated by the increased ability to neutralize the low pH of the stomach content in the duodenum as measured by capsules that can measure the pH during passage along the GI tract [45]. Duodenum alkalization is mainly due to pancreas

functioning, since non-CF patients who underwent complete pancreatectomy also require pH neutralizing agents to improve ERT [46]. Still, despite that duodenum alkalization neared normal levels, no change in ERT usage has been documented in CFTR-restoring drug trials, suggesting that restoration of pancreatic function was still was insufficient.

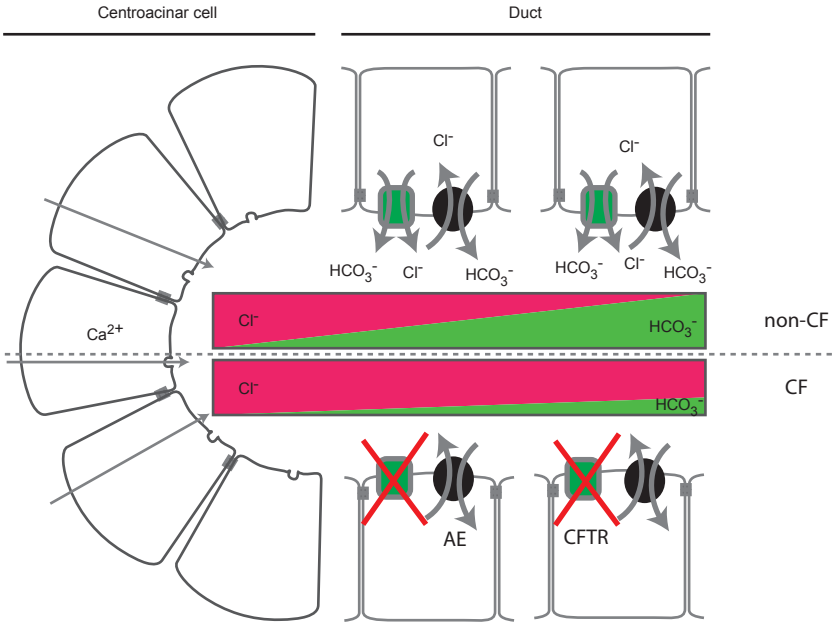

Figure 3. CFTR function in the pancreas. Initially CFTR is needed to drive the chloride/carbonate exchanger (AE, anion exchanger), later in the ducts, CFTR itself mediates carbonate secretion.

The Intestinal tract

The CF intestinal tract shows accumulation of sticky mucus, inflammatory lesions, and frequent intestinal obstructions. The altered mucus overlaying the intestinal epithelium impairs the innate barrier function in the intestine, and, similar as has been found in the ASL, defects have been reported in the activity of antimicrobial components in crypt secretions [47-50]. These likely contribute to impaired bacterial clearance compared with healthy individuals, leading to an altered composition of the intestinal microbiota in CF with respect to cell density, diversity and location [51]. It is very conceivable that the altered GI microbiota further contributes to GI problems such as small intestinal bacterial overgrowth, malnutrition, and intestinal inflammation [52].

Obstructions can occur in 15% of CF neonates where meconium accumulates in the ileum (meconium ileus, MI) requiring immediate surgery [53]. The danger of intestinal obstruction in infants is well recognized by Herman Boerhaave (Fig. 4), a Dutch professor at botany, chemistry and medicine [54]. In older CF patients, intestinal obstructions are referred to as distal intestine obstructive syndrome (DIOS), which can also occur within the small intestine [55]. Whereas MI and DIOS are both obstructions in the intestine, the underlying disease mechanism appear different since the occurance of DIOS is more related to environmental factors, whereas MI has a strong genetic component [56-57]. The

widespread dysfunction of CFTR along the entire GI tract, including the pancreatic and bile ducts results in a CF typical fatty stool, frequent abdominal pain and malnutrition of subjects with CF.

Figure 4. Herman Boerhaave (1668-1738) a Dutch professor at botany, medicine and chemistry already indicates the necessity to expel the meconium from the intestines. (Picture obtained from: Wellcome Library, London)

As observed in the nasal mucosa, electrophysical properties of the gut mucosa are severely affected in CF. Rectal biopsies are frequently isolated from patient to quantify chloride and bicarbonate ion transport, and especially in Europe these intestinal current measurements (ICM) are used in specialized centers for CF patients who are difficult-to-diagnose due to borderline diagnostic markers and genetic mutations of unknown consequence [58-61].

The sweat gland

The increased salt concentration in sweat is the classic diagnostic marker for CF [62]. References to the increased salt concentration can be found in literature dating back as far as 1500 [63] . This lead to the general beliefs that if a child's sweat tastes salty after being born, it is possessed and will therefore soon die [64-66]. Interestingly, in 1705, the pharmacists Johann Schmidt noted that children with salty sweat are most likely not possessed, but may suffer from an unknown underlying illness [66]. We now know that the secretory coil of sweat glands secrets isotonic fluid with a high chloride concentration. In healthy individuals the chloride is resorbed by CFTR along the sweat ducts, however, CF patients have vastly diminished chloride resorption resulting in a high chloride and sodium concentration in sweat (Fig. 5). An advantage of sweat chloride concentration as marker for CF disease is that this organ is not affected by mucus plugging and widespread inflammatory responses that can affect measurements of chloride transport in nasal or intestinal epithelium.

Other tissues

CFTR expression has been reported in various immune cell subsets [67-68], muscle tissue [69] and many other epithelia such as those forming the bile ducts [70] and vas deferens [71]. The increased survival of CF patients reveals an even more widespread and complex disease progression. Older people with CF show increased renal disease, drug allergies, mild hypothyroidism, CF-related diabetes, CF-related liver disease, increased bone absorption and intestinal cancer [72-74]. However, it remains to be investigated which of

these conditions is directly caused by dysfunctional CFTR and which are caused by the prolonged inflammation and drug induced.

Individual disease manifestations in CF

The impact of CFTR dysfunction on organ systems is highly variable between patients. Interactions between CF disease modifying genes, environmental factors and the *CFTR* gene itself at the level of the individual form the base of this variability [75]. The CF research community conducts a huge effort to define these CFTR genotype-phenotype relations to better predict the prognosis of disease for the individual patient.
Genetic research on CF twins and siblings is used to discriminate between genetic and environmental factors influencing CF disease progression. These studies identified genetic variation in the immune system and pulmonary system that contribute to disease variability [76-77]. Also the occurrence of diabetes and MI, both contributors to a detrimental disease progression, is attributed to genetic disease modifiers [75]. Large genetic modifier studies have been conducted, and are expanded is to further identify genetic modulators of disease [78-79].

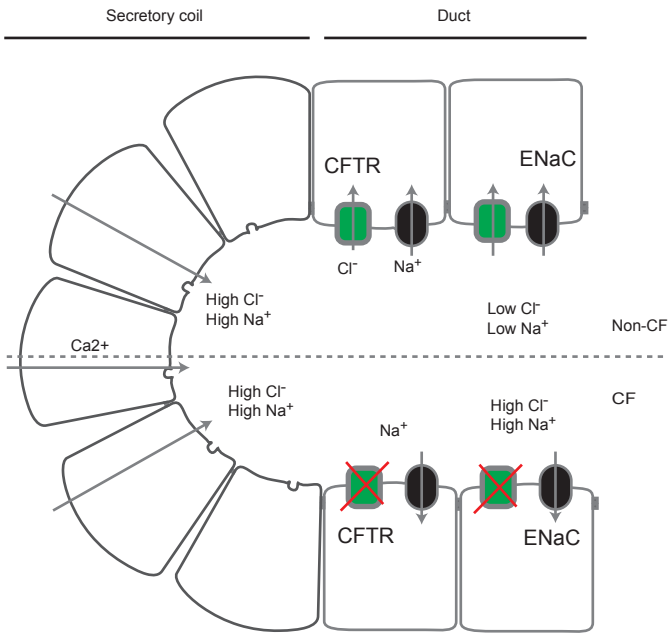

Figure 5. CFTR function in the sweat ducts. Absence of CFTR function in the sweat glands results in high sweat chloride concentration. The epithelial sodium channel (ENaC) functions less efficient without functional CFTR.

Being a monogenetic disease, the CF-causing mutations remain the largest contributors to disease, and mutations associated with significant residual CFTR function clearly show milder disease expression. However, even for patients with the same disease causing mutation, clear heterogeneity in disease phenotype and residual function has been observed [80]. These might be attributed to other genes influencing CFTR function, but (unidentified) *CFTR* polymorphisms can also contribute to increases or decreases in CFTR expression.

CFTR structure and activity

Based on the 1480 long amino acid sequence, CFTR is a member of the ATP binding cassette (ABC) transporter superfamily [81]. The CFTR protein consists of two multi membrane spanning domains (MSDs), two nucleotide binding domains (NBDs) and a regulatory domain (RD) [82] (Fig. 6). The MSDs form the channel pore and the NBDs are involved in ATP binding and hydrolysis, which regulates channel gating [83]. High-resolution crystal structure of the CFTR protein are lacking, but CFTR models based on the homologous bacterial efflux transporter Sav1866 or the human multidrug resistance protein 1 indicate that the transition from a closed to an open confirmation is energetically challenging, and that the F508del mutation further increases the energetic barrier to transition between these states [84].

Unlike other ABC family members, CFTR is a channel instead of a transporter facilitating rapid bidirectional flows of ions depending on concentration gradients and membrane potential. The RD is also unique for CFTR in comparison with ABC transporters. Phosphorylation of the RD is a prerequisite for channel activity. A total of 15 protein kinase A (PKA) sites have been identified in the RD, and removal of all these sites renders CFTR completely PKA-insensitive. Interestingly, two PKA sites that inhibit CFTR function upon phosphorylation might also be phosphorylated by AMP activated Kinase (AMPK) [85]. The RD of CFTR thus allows a tight control of cellular CFTR activity through cAMP-PKA-dependent signaling.

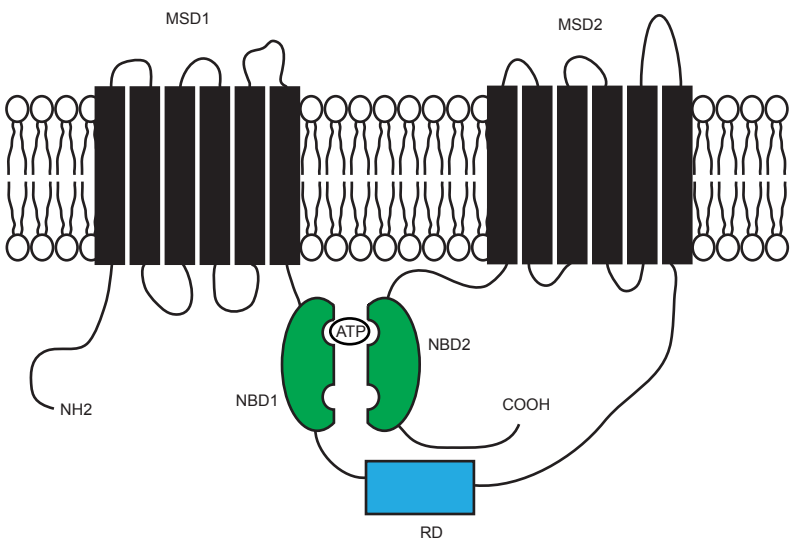

Figure 6. Global structure of CFTR. CFTR consists of 2 membrane spanning domains (MSD), which form the channel. Nucleotide binding domain (NBD) 1 and 2 are involved in opening and closing of the channel. The regulatory domain (RD) is a unique feature of CFTR, and regulates CFTR opening.

CFTR activation generally occurs via the cAMP-PKA pathway, but also protein kinase C (PKC) [86] and protein kinase G II (PKGII) can activate CFTR [87-88]. PKC is classically

activated via Ca^{2+} signaling, while PKG activation is induced by cGMP. The magnitude of CFTR activation via PKA, PKC and PKGII is likely to be tissue specific. For example, PKGII is not present in lung tissue but is expressed in the intestine [89]. Although CFTR can be activated via these alternate pathways, the classical cAMP-PKA-mediated CFTR activation route induces the most potent CFTR activation, and many tissues do not show clear CFTR function upon intracellular calcium signaling [90-91].

***In vivo* regulation of CFTR function**

Endogenous ligands control the amount of CFTR activation, which is different between tissues. CFTR activity in the intestine appears generally low as active infections that raise cAMP (e.g. by *V. cholera*) lead to secretory diarrhea. This would indicate that under endogenous conditions CFTR activity (rather than expression) is the rate limiting step in the intestine. In the GI tract, there are many mechanisms regulating CFTR-mediated fluid secretion such as vasoactive intestinal peptide (VIP) [91-93], PACAP-27 [94], prostaglandins [91], calcitonin [95] and histamine [96]. These ligands can induce cAMP-dependent CFTR activation. VIP and PACAP-27 are also capable of inducing Ca^{2+} signaling [94] and thus may also induce Ca^{2+}-mediated CFTR activation in the GI tract. Furthermore, substance P, and cholecystokinin induce Ca^{2+} signaling which can activate CFTR, in a PKC dependent manner [96]. Guanylin can induce cGMP-mediated CFTR activation in the intestine [91]. In the small intestine, the S cells secrete secretin due to a low pH or fatty acids [97], which induces cAMP-mediated [98] and CFTR-dependent bicarbonate secretion from the pancreas [39]. Also VIP can induce bicarbonate secretion from the pancreas [99]. In the bile ducts, CFTR is activated in a cAMP-dependent manner by bile salts [100] and secretin [101-103] thereby facilitating bile salt secretion into the GI tract. In cholangiocytes, VIP can induce bicarbonate secretion in a cAMP-independent manner [104], possibly using a Ca^{2+} dependent manner to activate CFTR [105]. Of note, the GI tract is divided into different sections, which could implicate that some fluid secreting molecules (secretagogues), only act in a particular section of the intestine.

In contrast, CFTR in the sweat ducts is suggested to be constitutively activated by cAMP-PKA-mediated signaling, with only a limited amount of isolated ducts that show additional responses to adrenergic stimuli [62,106-107]. For the airways, cAMP-mediated CFTR activation can be induced by adenosine and beta-adrenergic signaling [108-109] as well as apical UTP/ADP purinergic receptor agonists [110]. Whereas the PKC-mediated CFTR activation is observed for VIP [105,111-113], although the extent of PKC-mediated CFTR activation is difficult to determine, due to the presence of Ca^{2+}-activated chloride channels and cross talk between the calcium- and cAMP-pathways [114-116]. To what extent the entire pool of CFTR is activated by these stimuli is unclear, but NPD measurements would suggest that approximately 50% of CFTR is active in the upper airways, as an additional 50% of CFTR function can be induced by exogenous addition of adrenergic stimuli [117-118]. In the lower airways, it remains unclear, and might vary at different locations such as the submucosal gland, or CFTR-expressing surface epithelial cells. When CFTR activity is rate limited by cellular stimuli in the airways, supra-physiological stimulations of cellular pathways that activate CFTR may be beneficial for people with CF by maximizing CFTR activity.

1

CFTR mutations

Soon after the discovery of the *CFTR* gene it was recognized that many patients have the same mutation. The most prevalent mutation is the deletion of phenylalanine at position 508 (F508del), which is present in 87% of all patients on at least on allele [119]. However, an additional 2000 mutations have been described for which the functional consequences remain mostly unclear (www.CFTR2.org) [120]. Still, the disease liability has been established for ~130 CFTR mutations, which are carried by approximately 95% of subjects. However, for these mutations, the impact on CFTR function at the level of the individual still remains poorly indicated. To better define and characterize the large genetic heterogeneity observed in CFTR mutations, different mutations can be classified on their mechanism of CFTR disruption (Fig. 7). Although this classification system helps to categorize the large number of *CFTR* mutations, many mechanistic details on the less common mutations remain elusive, and new literature even suggest to use a classification model of ~30 variants [121]. This incorporates that mutations can belong to more than a single class, and also that residual function within a single class can differ significantly [122].

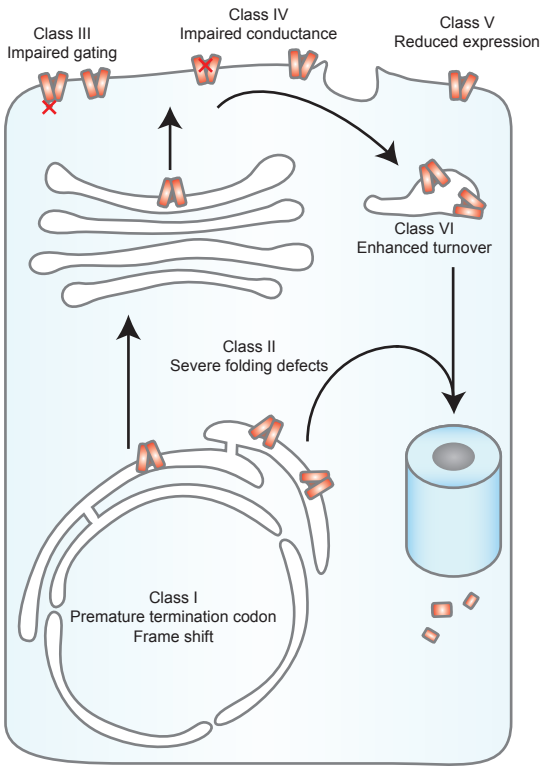

Figure 7. CFTR classification based on the mechanism of CFTR disruption. Class I mutations result in non functional protein synthesis. Class II mutations have severe folding defects resulting in premature degradation. Class III and IV mutations are expressed at the plasma membrane, but have channel opening and conductance defects, respectively. Class V and VI mutations have a reduced expression due either to impaired synthesis or enhanced protein turnover.

Class I mutations comprise nonsense mutations or premature termination codons (PTCs), deletions or insertions resulting in frame-shifts, and splice site mutations in the consensus site (+1, -1 of exon/intron boundaries). These mutations typically result in full loss of CFTR function, although that near-C-terminal mutations may be associated with

some function. Further, depending on the location of the nonsense mutation, the mRNA is a potential target for nonsense-mediated mRNA decay (NMD), which eliminates the nonsense containing mRNA [123]. Approximately 16% of the European CF patients have a class I mutation, however large variation between countries is observed [119]. Patients with two class I mutations generally are pancreas insufficient and have a high sweat chloride concentration.

Class II mutations severely disrupt CFTR folding and trafficking from the ER membrane to the apical membrane. These improperly folded proteins are recognized by quality control mechanisms and targeted for ER associated degradation [124-125]. The most common mutation, CFTR-F508del is an example of a class II mutation. Due to the high prevalence of the F508del-mutation, approximately 88**%** of all EU-patients have at least one class II mutation [119]. Patients with two class II mutations generally are pancreas insufficient, with a high sweat chloride concentration.

Class III mutations are expressed at the plasma membrane, but have a gating defect that severely reduces the function of the CFTR channel. The defective gating is caused by difficulties in ATP dependent channel opening and closing. The best described class III mutation, G551D, has the same clinical phenotype as the F508del mutation, with similar pancreas status and sweat chloride concentrations. Other mutations in this class are relative rare and literature about these mutations is scarce [126-131]. Approximately 4% of the CF patients have a class III mutation.

Class IV mutations are expressed at the plasma membrane but display reduced channel conductance due to mutations that affect the channel pore. The most common class IV mutation CFTR-R117H is associated with pancreas sufficiency, reflecting the relative mildness of this mutation, albeit that this mutation also has class III defects [132-133]. The CFTR-R117H is CF-causing when the mutation is in *cis* with polymorphisms in intron 8, which reduces the CFTR mRNA levels. The best described polymorphism described in intron intervening sequence (IVS) 8 is the poly-thymidine (T_n) repeat, near the 3' splice site of IVS 8, which can be 5, 7 or 9T long. A shorter T_n sequence results in exclusion of exon 9 from the mRNA. Exclusion of exon 9 does not reduce the overall level of CFTR mRNA, however a shorter CFTR protein is produced, which is misfolded and rapidly degraded [134-136]. An estimated 3% of the CF patients carry a class IV mutation, which is usually associated with pancreas sufficiency and intermittent sweat chloride levels. The residual CFTR function is also reflected by a slower annual decline in pulmonary function.

Class V mutations have a reduced number of normally functioning CFTR proteins at the plasma membrane. These include several alternative splice variants, such as 3849+10kb C->T [137] and 2789 +5G->A [138], and CFTR-A455E [139-140] mutation. These mutations are associated with a less severe disease phenotype and are present in 3% of the CF patients. Class VI mutants have a reduced retention of the protein at the plasma-membrane; an example of this is truncation at the C-terminal part of the protein. Although this group is rare, recent data on pharmacologically rescued of F508del indicates reduced plasma membrane retention of the F508del [141-142]. These mutations are associated with severe disease.
Generally, CFTR mutations belonging to class IV-VI are less severe than class I-III mutations. Patients with 2 class one mutations have a decreased FEV_1 compared to patients without a class I mutation [143]. This suggests that the mutation which is least impaired in function determines the clinical outcome.

1

CFTR modulating drugs

The first registered drug that targets and repairs function of the mutant CFTR protein directly is ivacaftor (VX-770). Currently, several drugs that can directly modulate CFTR function are being investigated in clinical trials, including VX-770, VX-809 (lumacaftor) and VX-661 (Fig. 8). These compounds aim to restore CFTR function by mechanically forcing the mutant CFTR into a conformation associated with enhanced function, and show mutation- and patient-dependent efficacy.

Two mechanistically distinct direct CFTR-targeting drugs exist, i.e. i) potentiators that increase the channel function of the apical CFTR protein by facilitating the channel open probability, and ii) correctors that enhance mutant CFTR trafficking to the cell surface. The first registered potentiator for use in the treatment of CF is ivacaftor, and is now proven effective for a variety of class III gating mutations (G178R, S549N, S549R, G551D, G551S, G1244E, S1251N, S1255P and G1349D) [144-146]. It was first registered for the most common gating mutation G551D, and clinical administration induces rapid improvement in FEV_1, body mass index (BMI) and sweat chloride and the intestinal pH is normalized [44]. Currently, efforts focus on the identification of new CFTR mutations or patient subgroups that benefit from VX-770 treatment [147]. These studies primarily focus on patients who have mutations associated with residual function such as the CFTR-R117H [148-149] or disease associated with milder symptoms [150]. VX-770 also increases CFTR function of the trafficking mutant F508del *in vitro* [151], but was not effective as mono-therapy for F508del patients [152].

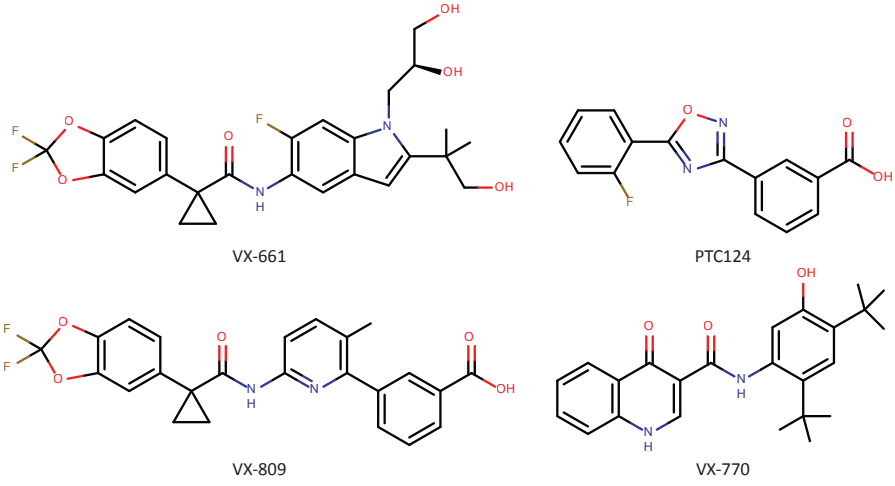

Figure 8. Chemical structures of the CFTR modulating compounds VX-661, PTC124, VX-809 and VX-770.

Correctors such as VX-809 and its successor VX-661 aim to increase plasma membrane trafficking of CFTR-F508del and thereby repair CFTR function. However, correction alone of the most common mutation CFTR-F508del was not sufficient for clinical improvement [153]. To further enhance function of corrected F508del protein at the plasma membrane, the opening of the channel can be further potentiated by VX-770 [154]. Indeed a phase II trial using a combination of VX-809 and VX-770 (okrambi) in patients homozygous

for F508del did show a modest increase in FEV_1 [155] which was confirmed in a larger phase III trial [156]. Data on VX-661 are still limited, but based on press releases made by Vertex it seems to improve lung function in F508del patients when combined with VX-770 compared to placebo [157-158]. Furthermore, *in vitro* data suggests that different correctors can synergize and thereby further increase CFTR function [159].

PTC124 (ataluren) is another CFTR modulating therapy is under development for premature termination codons. PTC124 does not directly target the CFTR protein, but rather the ribosomes that translate the CFTR mRNA into a functional protein and increase CFTR expression and function by inducing translational read-through when premature termination codons are encountered. Nonsense mutation suppression has been described for various compounds, such as G418 and gentamicin [160]. These findings initiated the development of PTC124 as non-toxic suppressor of premature termination codons [161]. Compounds that can induce PTC read-through are not very efficient and the attracted near cognate aminoacyl-tRNA is incorporated in order to continue, may affect function of the read-through product. Open-label phase II trials [162-164] showed only differences in nasal potential difference (NPD) upon treatment, while FEV_1 remained unchanged. However, in a phase III trial, ataluren neither improved NPD nor pulmonary function. Beneficial clinical effects were observed when patients are not using tobramycin, since this may affect the binding of PTC124 to ribosomes [165]. In addition, potentiators may further enhance treatment effects of nonsense suppressing compounds as suggested by *in vitro* studies [147,166].

Despite these exciting developments, low overall efficacy of these compounds and patient-to-patient variability limits robust treatment of the basic defect for the majority of patients. More effective therapies for F508del will most likely require the application of multiple correctors to robustly repair the multiple folding defects and plasma membrane trafficking [159,167-168]. Furthermore, the corrected F508del is destabilized at the plasma membrane by the current potentiator VX-770 indicating a need for additional potentiators [169-170]. As observed for correctors, combinations of mechanistically distinct potentiators may also be used to better repair the CFTR defect [171-172]. However, until now it remains difficult to predict if (combinations of) compounds will enable the restoration of CFTR function up to levels associated with no disease. It is therefore important to develop more effective compounds and complementary strategies for the restoration of CFTR function *in vivo*.

CFTR-dependent model systems for drug discovery

A variety of cellular models have been used to identify compounds that modulate CFTR. One of these approaches utilizes a genetically encoded halide-sensitive yellow fluorescent protein (YFP) [173-174]. These YFP variants can be quenched by iodide, which is efficiently transported into the cells via activated CFTR channels. This approach is especially suited for high throughput screening [168,175-178]. An alternative approach is the use of chemical dyes, which respond to changes in membrane potential [179] due to CFTR activation. Nonfunctional screening methods have also been used to screen for compounds that increase CFTR plasma membrane expression, which rely on extracellular epitope tagged CFTR [167,180]. However, the increase in CFTR expression does not always correlate with function, indicating the requirement for additional functional measurements.

Likewise, screens can be performed using purified mutant CFTR protein domains as readout [181].

The validation of hits identified in screens requires the identification of mechanisms of action and efficacy studies in human primary tissues and animal models [182-184]. The most validated human primary cell model to establish efficacy of hits are human bronchial cells cultured at an air-liquid interface. CFTR-dependent currents in these monolayers can be assessed by Ussing chamber experiments, which is often used to study ion transport across epithelial cells. Agonist induced channel activity combined with ion replacement is used to asses the activity of different ion channels, such as CFTR can be assessed. CFTR activity is measured by addition of cAMP-raising agents after inhibition of ENaC using amiloride. To establish the impact of compounds on single channel activity, patch-clamp technology is used [185] which is highly suited to evaluate changes in channel open-probabilities. Recently, a novel CFTR assay in a primary cell model system of intestinal stem cells was developed [186-187]. The intestinal stem cells are grown in 3D and form so-called organoids consisting of a single epithelial polarized layer surrounding an internal lumen where CFTR is expressed at the apical membrane [188]. The activation of CFTR in these organoids results in rapid CFTR-dependent influx of water and electrolytes, thereby increasing the overall size of these organoids. This model can be used to identify CFTR modulating drugs. Although this model has yet to be used in large scale high through-put screenings, it has the potential to be used in such screenings. Another potential application of this method is to discriminate between responders and non-responders to specific drugs [189].

Relevant animal models play a pivotal role in preclinical drug development. For CF, a variety of animal models have been generated, but their role for human drug development has been limited thus far. The murine CFTR-F508del has clearly different properties when compared with human F508del [190], and these mice do not exhibit a clear CF lung phenotype such as observed in other animal models such as the “βENaC” transgenic mouse [191]. The CF ferret model is still being explored and until now only a knock-out model exist [192]. Currently it appears that the CF pig model is the best model, albeit that it also only partially reflects human disease [193]. It develops spontaneous lung disease and infections, but due to its size it is an expensive animal model, and compounds need to be evaluated on pig F508del *in vitro* to address species-specific effects.

Identifying indirect modulators of CFTR function

The efficacy of the recent CFTR-targeting drugs remains limited for the majority of patients, so the need for parallel efforts to enhance their efficacy remains high. One of the hallmarks of CF is a chronic inflammatory process fueled by chronic bacterial and viral infections that ultimately result in tissue damage and pulmonary failure (Fig. 9) [194-196]. The inflammatory response thus plays a dual role in CF disease: on the one hand, inflammatory responses are essential to limit the impact of pathogen spread and infection, on the other hand chronic inflammation induces tissue remodeling and organ dysfunction. Ideally, inflammatory interventions in CF may focus on limiting the impact of chronic inflammation, while retaining pathogen defense.

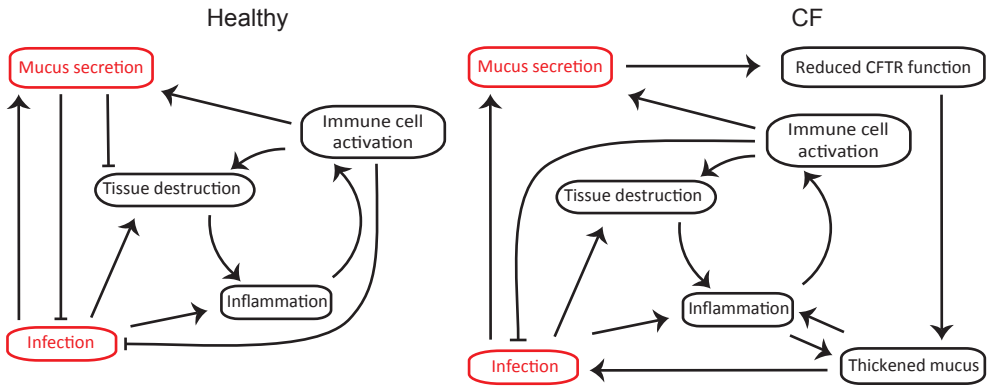

Figure 9. Interplay between pathogens and the immune system in a healthy and CF airways.

Currently, we do not know whether inflammatory pathways can be targeted to limit inflammation-induced chronic tissue remodeling, while not affecting pathogen defense. Recent literature indicate that CFTR is down-modulated by inflammatory cytokines, and points out that other chronic lung diseases such as COPD associate with low CFTR expression [197-198]. Now that CFTR-targeting compounds slowly become available for CF patients, inflammatory cytokines that down regulate CFTR can limit the *in vivo* efficacy of CFTR-restoration approaches. Indeed, some cytokines have already been found to down-modulate CFTR expression, but only few inflammatory mediators have been assessed for CFTR-modulating activity, such as IFN-γ [199] and TGF-β [200]. Intervention at the level of cytokines that modulate CFTR expression or function may be used to increase CFTR function in people with CF.

Another approach to enhance CFTR function *in vivo* may involve the activation of signaling that enhance the opening of the CFTR channel. As indicated above, cAMP-dependent signaling regulates CFTR activity, however, to what extent CFTR function is rate-limited by cAMP is mostly unknown and likely organ-specific. Such an approach would be useful in patients with CFTR residual function, either genetically encoded or drug-induced, when CFTR activity can be stimulated without too much systemic side effects of enhanced cAMP activity.

It could very well be that existing drugs on the market for non-CF indications may be repurposed for CF. In this way, novel therapeutic strategies can be rapidly implemented for treatment of a rare disease, for which drug development is economically and technically challenging. Crucial to such an approach would be a model to identify drugs that (indirectly) modulate CFTR, and the identification of patients that would benefit from such therapy. When these drugs modify CFTR function via indirect mechanisms, it is likely that these may further enhance the efficacy of direct CFTR-targeting drugs.

Current challenges in cystic fibrosis

The challenge in the field of cystic fibrosis remains the curative treatment of all patients. Due to the heterogeneity in CFTR mutations, different treatments will be needed: PTC-suppressing medication for nonsense mutations, potentiators for gating mutants, and correctors for trafficking mutants. Although these compounds are promising, the latest phase III PTC124 [165] and phase III VX-809/VX-770 [156] clinical trials did not yield the same improvements in clinical functions as did the VX-770 trial with G551D patients [145]. This would indicate that more research is needed to improve the efficacy of these compounds or that additional compounds are needed to achieve greater clinical efficacy. Symptomatic treatment strategies, such as mucolytics are also important for improving the life expectancy of all CF patients. Although not a cure for CF, symptomatic treatment can be easily implemented and act independent of the CFTR mutation. Furthermore, for difficult to treat mutations (due to the lack of available CFTR restoring compounds) symptomatic treatment might be the only solution. Gene-therapy can restore CFTR function throughout in the human body, although targeting every CFTR expressing tissue will remain challenging. Targeting the respiratory tract is more feasible, but still remains difficult as indicated by the results from transient non-viral gene replacement in the airways [201]. In the search for novel therapeutics, efficient models (e.g. cell lines) are needed in the identification process, whereas primary cell models are used to validate these initial hits and better translate to *in vivo* efficacy. Personalized strategies may help to further enhance the effects of treatments for patients that poorly respond, but will rely on drug-responsive biomarkers that correlate with long-term clinical outcome.

Out line of this thesis:

This scope of this thesis is to develop new methods that can assist the development of new CFTR modulators, and the application of such modulators in a more personalized setting. Chapter 2 describes the development of a novel YFP fusion protein with enhanced potential for high throughput screening. Chapter 3 shows how intestinal organoids can be used for analysis of drugs affecting premature termination codon readthrough. In Chapter 4, we develop methodology that can be used to quantitate circulating levels of CFTR modulators in plasma. Chapter 5 indicates that intestinal organoids can be used to screen small drug libraries for their potential to activate CFTR *in vitro* and *in vivo*. In chapter 6, we sought to identify inflammatory modulators that may regulate CFTR expression and function. Chapter 7 provides a discussion of chapters 2-6.

Reference list

1 Riordan, J. R. *et al.* Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066-1073, (1989).

2 Rommens, J. M. *et al.* Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* **245**, 1059-1065, (1989).

3 Kerem, B. *et al.* Identification of the cystic fibrosis gene: genetic analysis. *Science* **245**, 1073-1080, (1989).

4 Davis, P. B. Cystic fibrosis since 1938. *Am J Respir Crit Care Med* **173**, 475-482, (2006).

5 MacKenzie, T. *et al.* Longevity of patients with cystic fibrosis in 2000 to 2010 and beyond: survival analysis of the Cystic Fibrosis Foundation patient registry. *Ann Intern Med* **161**, 233-241, (2014).
6 Que, C., Cullinan, P. & Geddes, D. Improving rate of decline of FEV1 in young adults with cystic fibrosis. *Thorax* **61**, 155-157, (2006).
7 Voynow, J. A. & Rubin, B. K. Mucins, mucus, and sputum. *Chest* **135**, 505-512, (2009).
8 Cohen-Cymberknoh, M., Kerem, E., Ferkol, T. & Elizur, A. Airway inflammation in cystic fibrosis: molecular mechanisms and clinical implications. *Thorax* **68**, 1157-1162, (2013).
9 Folkesson, A. *et al.* Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. *Nat Rev Microbiol* **10**, 841-851, (2012).
10 Lyczak, J. B., Cannon, C. L. & Pier, G. B. Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* **15**, 194-222, (2002).
11 Christie, J. D. *et al.* The Registry of the International Society for Heart and Lung Transplantation: 29th adult lung and heart-lung transplant report-2012. *J Heart Lung Transplant* **31**, 1073-1086, (2012).
12 Sands, D. Transepithelial nasal potential difference (NPD) measurements in cystic fibrosis (CF). *Med Wieku Rozwoj* **17**, 13-17, (2013).
13 Bangel, N., Dahlhoff, C., Sobczak, K., Weber, W. M. & Kusche-Vihrog, K. Upregulated expression of ENaC in human CF nasal epithelium. *J Cyst Fibros* **7**, 197-205, (2008).
14 Loebinger, M. R., Bilton, D. & Wilson, R. Upper airway 2: Bronchiectasis, cystic fibrosis and sinusitis. *Thorax* **64**, 1096-1101, (2009).
15 Jorissen, M. B., De Boeck, K. & Cuppens, H. Genotype-phenotype correlations for the paranasal sinuses in cystic fibrosis. *Am J Respir Crit Care Med* **159**, 1412-1416, (1999).
16 Mainz, J. G. *et al.* Concordant genotype of upper and lower airways P aeruginosa and S aureus isolates in cystic fibrosis. *Thorax* **64**, 535-540, (2009).
17 van der Gast, C. J. *et al.* Three clinically distinct chronic pediatric airway infections share a common core microbiota. *Ann Am Thorac Soc* **11**, 1039-1048, (2014).
18 Prevaes, S. M. *et al.* Development of the Nasopharyngeal Microbiota in Infants with Cystic Fibrosis. *Am J Respir Crit Care Med*, (2015).
19 Hoegger, M. J. *et al.* Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis. *Science* **345**, 818-822, (2014).
20 Adam, R. J. *et al.* Air trapping and airflow obstruction in newborn cystic fibrosis piglets. *Am J Respir Crit Care Med* **188**, 1434-1441, (2013).
21 Choi, J. Y. *et al.* Aberrant CFTR-dependent HCO3- transport in mutations associated with cystic fibrosis. *Nature* **410**, 94-97, (2001).
22 Chen, E. Y., Yang, N., Quinton, P. M. & Chin, W. C. A new role for bicarbonate in mucus formation. *Am J Physiol Lung Cell Mol Physiol* **299**, L542-549, (2010).
23 Gustafsson, J. K. *et al.* Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype. *J Exp Med* **209**, 1263-1272, (2012).
24 Quinton, P. M. Cystic fibrosis: impaired bicarbonate secretion and mucoviscidosis. *Lancet* **372**, 415-417, (2008).
25 Yang, N., Garcia, M. A. & Quinton, P. M. Normal mucus formation requires cAMP-dependent HCO3- secretion and Ca2+-mediated mucin exocytosis. *J Physiol* **591**, 4581-4593, (2013).
26 Birket, S. E. *et al.* A functional anatomic defect of the cystic fibrosis airway. *Am J Respir Crit Care Med* **190**, 421-432, (2014).
27 Livraghi-Butrico, A. *et al.* Mucus clearance, MyD88-dependent and MyD88-independent immunity modulate lung susceptibility to spontaneous bacterial infection and inflammation. *Mucosal Immunol* **5**, 397-408, (2012).
28 Keiser, N. W. *et al.* Defective innate immunity and hyperinflammation in newborn cystic fibrosis transmembrane conductance regulator-knockout ferret lungs. *Am J Respir Cell Mol Biol* **52**, 683-694, (2015).
29 Wine, J. J. The development of lung disease in cystic fibrosis pigs. *Sci Transl Med* **2**, 29ps20, (2010).
30 Pezzulo, A. A. *et al.* Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature* **487**, 109-113, (2012).
31 Stoltz, D. A. *et al.* Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med* **2**, 29ra31, (2010).
32 Shah, V. S. *et al.* Airway acidification initiates host defense abnormalities in cystic fibrosis mice. *Science* **351**, 503-507, (2016).
33 Allen, D. B. *et al.* Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms. *Arch Surg* **132**, 991-996, (1997).
34 Bidani, A. & Heming, T. A. Effects of bafilomycin A1 on functional capabilities of LPS-activated alveolar macrophages. *J Leukoc Biol* **57**, 275-281, (1995).

35 Clary-Meinesz, C., Mouroux, J., Cosson, J., Huitorel, P. & Blaive, B. Influence of external pH on ciliary beat frequency in human bronchi and bronchioles. *Eur Respir J* **11**, 330-333, (1998).

36 Knowles, M. R. & Boucher, R. C. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest* **109**, 571-577, (2002).

37 Bartholin, T. *Historiarum anatomicarum rariorum, centuria III et IV*. (Comitis Adrianus Vlacq, 1657).

38 Littlewood, J. *http://www.cfmedicine.com/history/topics/pathology.htm*. (2014).

39 Ishiguro, H. *et al.* Physiology and pathophysiology of bicarbonate secretion by pancreatic duct epithelium. *Nagoya J Med Sci* **74**, 1-18, (2012).

40 Wilschanski, M. & Novak, I. The cystic fibrosis of exocrine pancreas. *Cold Spring Harb Perspect Med* **3**, a009746, (2013).

41 Brennan, A. L. & Beynon, J. Clinical updates in cystic fibrosis-related diabetes. *Semin Respir Crit Care Med* **36**, 236-250, (2015).

42 Vernooij-van Langen, A. M. *et al.* Novel strategies in newborn screening for cystic fibrosis: a prospective controlled study. *Thorax* **67**, 289-295, (2012).

43 Fieker, A., Philpott, J. & Armand, M. Enzyme replacement therapy for pancreatic insufficiency: present and future. *Clin Exp Gastroenterol* **4**, 55-73, (2011).

44 Rowe, S. M. *et al.* Clinical mechanism of the cystic fibrosis transmembrane conductance regulator potentiator ivacaftor in G551D-mediated cystic fibrosis. *Am J Respir Crit Care Med* **190**, 175-184, (2014).

45 Gelfond, D., Ma, C., Semler, J. & Borowitz, D. Intestinal pH and gastrointestinal transit profiles in cystic fibrosis patients measured by wireless motility capsule. *Dig Dis Sci* **58**, 2275-2281, (2013).

46 Kahl, S. & Malfertheiner, P. Exocrine and endocrine pancreatic insufficiency after pancreatic surgery. *Best Pract Res Clin Gastroenterol* **18**, 947-955, (2004).

47 Hansson, G. C. Role of mucus layers in gut infection and inflammation. *Curr Opin Microbiol* **15**, 57-62, (2012).

48 Munck, A. Cystic fibrosis: evidence for gut inflammation. *Int J Biochem Cell Biol* **52**, 180-183, (2014).

49 Ehre, C., Ridley, C. & Thornton, D. J. Cystic fibrosis: an inherited disease affecting mucin-producing organs. *Int J Biochem Cell Biol* **52**, 136-145, (2014).

50 Clarke, L. L. *et al.* Abnormal Paneth cell granule dissolution and compromised resistance to bacterial colonization in the intestine of CF mice. *Am J Physiol Gastrointest Liver Physiol* **286**, G1050-1058, (2004).

51 Werlin, S. L. *et al.* Evidence of intestinal inflammation in patients with cystic fibrosis. *J Pediatr Gastroenterol Nutr* **51**, 304-308, (2010).

52 Singh, V. V. & Toskes, P. P. Small bowel bacterial overgrowth: presentation, diagnosis, and treatment. *Curr Gastroenterol Rep* **5**, 365-372, (2003).

53 van der Doef, H. P., Kokke, F. T., van der Ent, C. K. & Houwen, R. H. Intestinal obstruction syndromes in cystic fibrosis: meconium ileus, distal intestinal obstruction syndrome, and constipation. *Curr Gastroenterol Rep* **13**, 265-270, (2011).

54 Boerhaave, H. *A Treatise on the Powers of Medicines*. (John Wilcox, 1740).

55 Farrelly, P. J., Charlesworth, C., Lee, S., Southern, K. W. & Baillie, C. T. Gastrointestinal surgery in cystic fibrosis: a 20-year review. *J Pediatr Surg* **49**, 280-283, (2014).

56 Blackman, S. M. *et al.* Relative contribution of genetic and nongenetic modifiers to intestinal obstruction in cystic fibrosis. *Gastroenterology* **131**, 1030-1039, (2006).

57 Stoltz, D. A. *et al.* Intestinal CFTR expression alleviates meconium ileus in cystic fibrosis pigs. *J Clin Invest* **123**, 2685-2693, (2013).

58 Cohen-Cymberknoh, M. *et al.* Evaluation of the intestinal current measurement method as a diagnostic test for cystic fibrosis. *Pediatr Pulmonol* **48**, 229-235, (2013).

59 Derichs, N. *et al.* Intestinal current measurement for diagnostic classification of patients with questionable cystic fibrosis: validation and reference data. *Thorax* **65**, 594-599, (2010).

60 Hug, M. J. & Tummler, B. Intestinal current measurements to diagnose cystic fibrosis. *J Cyst Fibros* **3 Suppl 2**, 157-158, (2004).

61 Sousa, M. *et al.* Measurements of CFTR-mediated Cl- secretion in human rectal biopsies constitute a robust biomarker for Cystic Fibrosis diagnosis and prognosis. *PLoS One* **7**, e47708, (2012).

62 Quinton, P. M. Cystic fibrosis: lessons from the sweat gland. *Physiology (Bethesda)* **22**, 212-225, (2007).

63 *Liber incantationum, exorcismorum et fascinationum variarum - BSB Clm 849*.

64 de Fontecha, J. A. *Diez previlegios para mugeres prenadas. Con un diccionario medico*. (Luys Martinez Grande, 1606).

65 Littlewood, J. http://www.cfmedicine.com/history/earlyyears.htm. (2014).
66 Schmidt, J. G. *Die gestriegelte Rocken-Philosophia, oder auffrichtige Untersuchung derer von vielen super-klugen Weibern hochgehaltenen Aberglauben*. Vol. 1 (Conrad Stössel, 1705).
67 Ettorre, M. *et al.* Electrophysiological evaluation of cystic fibrosis conductance transmembrane regulator (CFTR) expression in human monocytes. *Biochim Biophys Acta* **1840**, 3088-3095, (2014).
68 Sorio, C. *et al.* Defective CFTR expression and function are detectable in blood monocytes: development of a new blood test for cystic fibrosis. *PLoS One* **6**, e22212, (2011).
69 Bulley, S. & Jaggar, J. H. Cl(-) channels in smooth muscle cells. *Pflugers Arch* **466**, 861-872, (2014).
70 Concepcion, A. R., Lopez, M., Ardura-Fabregat, A. & Medina, J. F. Role of AE2 for pHi regulation in biliary epithelial cells. *Front Physiol* **4**, 413, (2013).
71 Marcorelles, P. *et al.* Cystic fibrosis transmembrane conductance regulator protein expression in the male excretory duct system during development. *Hum Pathol* **43**, 390-397, (2012).
72 Plant, B. J., Goss, C. H., Plant, W. D. & Bell, S. C. Management of comorbidities in older patients with cystic fibrosis. *Lancet Respir Med* **1**, 164-174, (2013).
73 Li, H., Ganta, S. & Fong, P. Altered ion transport by thyroid epithelia from CFTR(-/-) pigs suggests mechanisms for hypothyroidism in cystic fibrosis. *Exp Physiol* **95**, 1132-1144, (2010).
74 Naehrlich, L., Dorr, H. G., Bagheri-Behrouzi, A. & Rauh, M. Iodine deficiency and subclinical hypothyroidism are common in cystic fibrosis patients. *J Trace Elem Med Biol* **27**, 122-125, (2013).
75 Cutting, G. R. Cystic fibrosis genetics: from molecular understanding to clinical application. *Nat Rev Genet* **16**, 45-56, (2015).
76 Collaco, J. M. & Cutting, G. R. Update on gene modifiers in cystic fibrosis. *Curr Opin Pulm Med* **14**, 559-566, (2008).
77 Chalmers, J. D., Fleming, G. B., Hill, A. T. & Kilpatrick, D. C. Impact of mannose-binding lectin insufficiency on the course of cystic fibrosis: A review and meta-analysis. *Glycobiology* **21**, 271-282, (2011).
78 Emond, M. J. *et al.* Exome sequencing of extreme phenotypes identifies DCTN4 as a modifier of chronic Pseudomonas aeruginosa infection in cystic fibrosis. *Nat Genet* **44**, 886-889, (2012).
79 Wright, F. A. *et al.* Genome-wide association and linkage identify modifier loci of lung disease severity in cystic fibrosis at 11p13 and 20q13.2. *Nat Genet* **43**, 539-546, (2011).
80 Kerem, B. & Kerem, E. The molecular basis for disease variability in cystic fibrosis. *Eur J Hum Genet* **4**, 65-73, (1996).
81 Riordan, J. R. CFTR function and prospects for therapy. *Annu Rev Biochem* **77**, 701-726, (2008).
82 Cant, N., Pollock, N. & Ford, R. C. CFTR structure and cystic fibrosis. *Int J Biochem Cell Biol* **52**, 15-25, (2014).
83 Frelet, A. & Klein, M. Insight in eukaryotic ABC transporter function by mutation analysis. *FEBS Lett* **580**, 1064-1084, (2006).
84 Sorum, B., Czege, D. & Csanady, L. Timing of CFTR Pore Opening and Structure of Its Transition State. *Cell* **163**, 724-733, (2015).
85 King, J. D., Jr. *et al.* AMP-activated protein kinase phosphorylation of the R domain inhibits PKA stimulation of CFTR. *Am J Physiol Cell Physiol* **297**, C94-101, (2009).
86 Picciotto, M. R., Cohn, J. A., Bertuzzi, G., Greengard, P. & Nairn, A. C. Phosphorylation of the cystic fibrosis transmembrane conductance regulator. *J Biol Chem* **267**, 12742-12752, (1992).
87 Tien, X. Y., Brasitus, T. A., Kaetzel, M. A., Dedman, J. R. & Nelson, D. J. Activation of the cystic fibrosis transmembrane conductance regulator by cGMP in the human colonic cancer cell line, Caco-2. *J Biol Chem* **269**, 51-54, (1994).
88 Selvaraj, N. G., Prasad, R., Goldstein, J. L. & Rao, M. C. Evidence for the presence of cGMP-dependent protein kinase-II in human distal colon and in T84, the colonic cell line. *Biochim Biophys Acta* **1498**, 32-43, (2000).
89 Greengard, P., Nairn, A. C., Shenolikar, S., Armstrong, D. L. & Rossie, S. *Ion Channel Regulation*. (Elsevier Science, 1999).
90 Berger, H. A., Travis, S. M. & Welsh, M. J. Regulation of the cystic fibrosis transmembrane conductance regulator Cl-channel by specific protein kinases and protein phosphatases. *J Biol Chem* **268**, 2037-2047, (1993).
91 Seidler, U. *et al.* A functional CFTR protein is required for mouse intestinal cAMP-, cGMP- and Ca(2+)-dependent HCO3- secretion. *J Physiol* **505 (Pt 2)**, 411-423, (1997).

92 Ekblad, E. & Sundler, F. Distinct receptors mediate pituitary adenylate cyclase-activating peptide- and vasoactive intestinal peptide-induced relaxation of rat ileal longitudinal muscle. *Eur J Pharmacol* **334**, 61-66, (1997).

93 Cooke, H. J. Neurotransmitters in neuronal reflexes regulating intestinal secretion. *Ann N Y Acad Sci* **915**, 77-80, (2000).

94 Dickson, L., Aramori, I., McCulloch, J., Sharkey, J. & Finlayson, K. A systematic comparison of intracellular cyclic AMP and calcium signalling highlights complexities in human VPAC/PAC receptor pharmacology. *Neuropharmacology* **51**, 1086-1098, (2006).

95 Liu, H. *et al.* Calcitonin receptor-mediated CFTR activation in human intestinal epithelial cells. *J Cell Mol Med* **15**, 2697-2705, (2011).

96 Wapnir, R. A. & Teichberg, S. Regulation mechanisms of intestinal secretion: implications in nutrient absorption. *J Nutr Biochem* **13**, 190-199, (2002).

97 Williams, J. A. Secretin. *Pancreapedia: Exocrine Pancreas Knowledge Base*, (2013).

98 Harikumar, K. G., Happs, R. M. & Miller, L. J. Dimerization in the absence of higher-order oligomerization of the G protein-coupled secretin receptor. *Biochim Biophys Acta* **1778**, 2555-2563, (2008).

99 Chappe, V. & Said, S. I. in *Cystic Fibrosis - Renewed Hopes Through Research* (ed D. Sriramulu) Ch. VIP as a Corrector of CFTR Trafficking and Membrane Stability, (InTech, 2012).

100 Bijvelds, M. J. *et al.* Activation of CFTR by ASBT-mediated bile salt absorption. *Am J Physiol Gastrointest Liver Physiol* **289**, G870-879, (2005).

101 Kanno, N., LeSage, G., Glaser, S. & Alpini, G. Regulation of cholangiocyte bicarbonate secretion. *Am J Physiol Gastrointest Liver Physiol* **281**, G612-625, (2001).

102 Banales, J. M., Prieto, J. & Medina, J. F. Cholangiocyte anion exchange and biliary bicarbonate excretion. *World J Gastroenterol* **12**, 3496-3511, (2006).

103 Lenzen, R., Alpini, G. & Tavoloni, N. Secretin stimulates bile ductular secretory activity through the cAMP system. *Am J Physiol* **263**, G527-532, (1992).

104 Cho, W. K. & Boyer, J. L. Vasoactive intestinal polypeptide is a potent regulator of bile secretion from rat cholangiocytes. *Gastroenterology* **117**, 420-428, (1999).

105 Chappe, F., Loewen, M. E., Hanrahan, J. W. & Chappe, V. Vasoactive intestinal peptide increases cystic fibrosis transmembrane conductance regulator levels in the apical membrane of Calu-3 cells through a protein kinase C-dependent mechanism. *J Pharmacol Exp Ther* **327**, 226-238, (2008).

106 Reddy, M. M. & Quinton, P. M. PKA mediates constitutive activation of CFTR in human sweat duct. *J Membr Biol* **231**, 65-78, (2009).

107 Reddy, M. M., Sun, D. & Quinton, P. M. Apical heterotrimeric g-proteins activate CFTR in the native sweat duct. *J Membr Biol* **179**, 51-61, (2001).

108 Com, G. & Clancy, J. P. Adenosine receptors, cystic fibrosis, and airway hydration. *Handb Exp Pharmacol*, 363-381, (2009).

109 Hentchel-Franks, K. *et al.* Activation of airway cl- secretion in human subjects by adenosine. *Am J Respir Cell Mol Biol* **31**, 140-146, (2004).

110 Kunzelmann, K. & Mall, M. Pharmacotherapy of the ion transport defect in cystic fibrosis: role of purinergic receptor agonists and other potential therapeutics. *Am J Respir Med* **2**, 299-309, (2003).

111 Qu, F. *et al.* Activation of CFTR trafficking and gating by vasoactive intestinal peptide in human bronchial epithelial cells. *J Cell Biochem* **112**, 902-908, (2011).

112 Derand, R. *et al.* Activation of VPAC1 receptors by VIP and PACAP-27 in human bronchial epithelial cells induces CFTR-dependent chloride secretion. *Br J Pharmacol* **141**, 698-708, (2004).

113 Joo, N. S. *et al.* Absent secretion to vasoactive intestinal peptide in cystic fibrosis airway glands. *J Biol Chem* **277**, 50710-50715, (2002).

114 Ahuja, M., Jha, A., Maleth, J., Park, S. & Muallem, S. cAMP and Ca(2)(+) signaling in secretory epithelia: crosstalk and synergism. *Cell Calcium* **55**, 385-393, (2014).

115 Hofer, A. M. Interactions between calcium and cAMP signaling. *Curr Med Chem* **19**, 5768-5773, (2012).

116 Cooper, D. M., Mons, N. & Karpen, J. W. Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature* **374**, 421-424, (1995).

117 Knowles, M. R., Paradiso, A. M. & Boucher, R. C. In vivo nasal potential difference: techniques and protocols for assessing efficacy of gene transfer in cystic fibrosis. *Hum Gene Ther* **6**, 445-455, (1995).

118 Knowles, M. R. *et al.* Abnormal ion permeation through cystic fibrosis respiratory epithelium. *Science* **221**, 1067-1070, (1983).

119 De Boeck, K., Zolin, A., Cuppens, H., Olesen, H. V. & Viviani, L. The relative frequency of CFTR mutation classes in European patients with cystic fibrosis. *J Cyst Fibros* **13**, 403-409, (2014).

120 Sosnay, P. R. *et al.* Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene. *Nat Genet* **45**, 1160-1167, (2013).

121 Veit, G. *et al.* From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations. *Mol Biol Cell* **27**, 424-433, (2016).

122 Zielenski, J. Genotype and phenotype in cystic fibrosis. *Respiration* **67**, 117-133, (2000).

123 Popp, M. W. & Maquat, L. E. Organizing principles of mammalian nonsense-mediated mRNA decay. *Annu Rev Genet* **47**, 139-165, (2013).

124 Rogan, M. P., Stoltz, D. A. & Hornick, D. B. Cystic fibrosis transmembrane conductance regulator intracellular processing, trafficking, and opportunities for mutation-specific treatment. *Chest* **139**, 1480-1490, (2011).

125 Farinha, C. M., Matos, P. & Amaral, M. D. Control of cystic fibrosis transmembrane conductance regulator membrane trafficking: not just from the endoplasmic reticulum to the Golgi. *FEBS J* **280**, 4396-4406, (2013).

126 Castellani, C. *et al.* Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. *J Cyst Fibros* **7**, 179-196, (2008).

127 Illek, B. *et al.* Defective function of the cystic fibrosis-causing missense mutation G551D is recovered by genistein. *Am J Physiol* **277**, C833-839, (1999).

128 Seibert, F. S. *et al.* Disease-associated mutations in cytoplasmic loops 1 and 2 of cystic fibrosis transmembrane conductance regulator impede processing or opening of the channel. *Biochemistry* **36**, 11966-11974, (1997).

129 Orozco, L., Lezana, J. L., Villarreal, M. T., Chavez, M. & Carnevale, A. Mild cystic fibrosis disease in three Mexican delta-F508/G551S compound heterozygous siblings. *Clin Genet* **47**, 96-98, (1995).

130 Lissens, W., Bonduelle, M., Malfroot, A., Dab, I. & Liebaers, I. A serine to proline substitution (S1255P) in the second nucleotide binding fold of the cystic fibrosis gene. *Hum Mol Genet* **1**, 441-442, (1992).

131 Seibert, F. S. *et al.* Cytoplasmic loop three of cystic fibrosis transmembrane conductance regulator contributes to regulation of chloride channel activity. *J Biol Chem* **271**, 27493-27499, (1996).

132 Sheppard, D. N. *et al.* Mutations in CFTR associated with mild-disease-form Cl- channels with altered pore properties. *Nature* **362**, 160-164, (1993).

133 Loubieres, Y. *et al.* Association between genetically determined pancreatic status and lung disease in adult cystic fibrosis patients. *Chest* **121**, 73-80, (2002).

134 Chu, C. S., Trapnell, B. C., Curristin, S., Cutting, G. R. & Crystal, R. G. Genetic basis of variable exon 9 skipping in cystic fibrosis transmembrane conductance regulator mRNA. *Nat Genet* **3**, 151-156, (1993).

135 Zielenski, J. *et al.* CFTR gene variant for patients with congenital absence of vas deferens. *Am J Hum Genet* **57**, 958-960, (1995).

136 Groman, J. D. *et al.* Variation in a repeat sequence determines whether a common variant of the cystic fibrosis transmembrane conductance regulator gene is pathogenic or benign. *Am J Hum Genet* **74**, 176-179, (2004).

137 Dreyfus, D. H., Bethel, R. & Gelfand, E. W. Cystic fibrosis 3849+10kb C > T mutation associated with severe pulmonary disease and male fertility. *Am J Respir Crit Care Med* **153**, 858-860, (1996).

138 Dugueperoux, I. & De Braekeleer, M. The CFTR 3849+10kbC->T and 2789+5G->A alleles are associated with a mild CF phenotype. *Eur Respir J* **25**, 468-473, (2005).

139 Gan, K. H. *et al.* A cystic fibrosis mutation associated with mild lung disease. *N Engl J Med* **333**, 95-99, (1995).

140 De Braekeleer, M., Allard, C., Leblanc, J. P., Simard, F. & Aubin, G. Genotype-phenotype correlation in cystic fibrosis patients compound heterozygous for the A455E mutation. *Hum Genet* **101**, 208-211, (1997).

141 He, L. *et al.* Correctors of DeltaF508 CFTR restore global conformational maturation without thermally stabilizing the mutant protein. *FASEB J* **27**, 536-545, (2013).

142 Kotha, K. & Clancy, J. P. Ivacaftor treatment of cystic fibrosis patients with the G551D mutation: a review of the evidence. *Ther Adv Respir Dis* **7**, 288-296, (2013).

143 Geborek, A. & Hjelte, L. Association between genotype and pulmonary phenotype in cystic fibrosis patients with severe mutations. *J Cyst Fibros* **10**, 187-192, (2011).

144 Davies, J. C. *et al.* Efficacy and safety of ivacaftor in patients aged 6 to 11 years with cystic fibrosis with a G551D mutation. *Am J Respir Crit Care Med* **187**, 1219-1225, (2013).
145 Ramsey, B. W. *et al.* A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *N Engl J Med* **365**, 1663-1672, (2011).
146 Yu, H. *et al.* Ivacaftor potentiation of multiple CFTR channels with gating mutations. *J Cyst Fibros* **11**, 237-245, (2012).
147 Van Goor, F., Yu, H., Burton, B. & Hoffman, B. J. Effect of ivacaftor on CFTR forms with missense mutations associated with defects in protein processing or function. *J Cyst Fibros* **13**, 29-36, (2014).
148 Vertex Pharmaceuticals Incorporated. *Vertex Submits Supplemental New Drug Application (sNDA) to U.S. Food and Drug Administration for Use of KALYDECO® (ivacaftor) in People 18 and Older with Cystic Fibrosis who have the R117H Mutation.*, 2014).
149 Vertex Pharmaceuticals Incorporated. *U.S. Food and Drug Administration Approves KALYDECO® (ivacaftor) for Use in People with Cystic Fibrosis Ages 6 and Older Who Have the R117H Mutation*, 2014).
150 Vertex Pharmaceuticals Incorporated. *Proof-of-Concept Study of Ivacaftor Monotherapy Showed Improvements in Lung Function After Two Weeks of Treatment in People with Cystic Fibrosis who have a Residual Function Mutation.*, 2014).
151 Van Goor, F. *et al.* Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A* **106**, 18825-18830, (2009).
152 Flume, P. A. *et al.* Ivacaftor in subjects with cystic fibrosis who are homozygous for the F508del-CFTR mutation. *Chest* **142**, 718-724, (2012).
153 Clancy, J. P. *et al.* Results of a phase IIa study of VX-809, an investigational CFTR corrector compound, in subjects with cystic fibrosis homozygous for the F508del-CFTR mutation. *Thorax* **67**, 12-18, (2012).
154 Dalemans, W. *et al.* Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. *Nature* **354**, 526-528, (1991).
155 Boyle, M. P. *et al.* A CFTR corrector (lumacaftor) and a CFTR potentiator (ivacaftor) for treatment of patients with cystic fibrosis who have a phe508del CFTR mutation: a phase 2 randomised controlled trial. *Lancet Respir Med* **2**, 527-538, (2014).
156 Wainwright, C. E. *et al.* Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N Engl J Med* **373**, 220-231, (2015).
157 Vertex Pharmaceuticals Incorporated. *Addition of VX-661 to KALYDECO® (ivacaftor) Improves Lung Function in People with CF Who Are Heterozygous for the F508del and G551D Mutations in 28-day Phase 2 Proof-of-Concept Study.*, 2014).
158 Vertex Pharmaceuticals Incorporated. *Teatment with VX-661 and Ivacaftor in a Phase 2 Study Resulted in Statistically Significant Improvements in Lung Function in People with Cystic Fibrosis Who Have Two Copies of the F508del Mutation.*, 2014).
159 Okiyoneda, T. *et al.* Mechanism-based corrector combination restores DeltaF508-CFTR folding and function. *Nat Chem Biol* **9**, 444-454, (2013).
160 Howard, M., Frizzell, R. A. & Bedwell, D. M. Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. *Nat Med* **2**, 467-469, (1996).
161 Welch, E. M. *et al.* PTC124 targets genetic disorders caused by nonsense mutations. *Nature* **447**, 87-91, (2007).
162 Kerem, E. *et al.* Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial. *Lancet* **372**, 719-727, (2008).
163 Sermet-Gaudelus, I. *et al.* Ataluren (PTC124) induces cystic fibrosis transmembrane conductance regulator protein expression and activity in children with nonsense mutation cystic fibrosis. *Am J Respir Crit Care Med* **182**, 1262-1272, (2010).
164 Wilschanski, M. *et al.* Chronic ataluren (PTC124) treatment of nonsense mutation cystic fibrosis. *Eur Respir J* **38**, 59-69, (2011).
165 Kerem, E. *et al.* Ataluren for the treatment of nonsense-mutation cystic fibrosis: a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Respir Med* **2**, 539-547, (2014).
166 Xue, X. *et al.* Synthetic aminoglycosides efficiently suppress cystic fibrosis transmembrane conductance regulator nonsense mutations and are enhanced by ivacaftor. *Am J Respir Cell Mol Biol* **50**, 805-816, (2014).
167 Phuan, P. W. *et al.* Synergy-based small-molecule screen using a human lung epithelial cell line yields DeltaF508-CFTR correctors that augment VX-809 maximal efficacy. *Mol Pharmacol* **86**, 42-51, (2014).

168 Lin, S. *et al.* Identification of synergistic combinations of F508del cystic fibrosis transmembrane conductance regulator (CFTR) modulators. *Assay Drug Dev Technol* **8**, 669-684, (2010).
169 Veit, G. *et al.* Some gating potentiators, including VX-770, diminish DeltaF508-CFTR functional expression. *Sci Transl Med* **6**, 246ra297, (2014).
170 Cholon, D. M. *et al.* Potentiator ivacaftor abrogates pharmacological correction of DeltaF508 CFTR in cystic fibrosis. *Sci Transl Med* **6**, 246ra296, (2014).
171 Pyle, L. C. *et al.* Regulatory domain phosphorylation to distinguish the mechanistic basis underlying acute CFTR modulators. *Am J Physiol Lung Cell Mol Physiol* **301**, L587-597, (2011).
172 Sohma, Y., Yu, Y. C. & Hwang, T. C. Curcumin and genistein: the combined effects on disease-associated CFTR mutants and their clinical implications. *Curr Pharm Des* **19**, 3521-3528, (2013).
173 Galietta, L. V., Jayaraman, S. & Verkman, A. S. Cell-based assay for high-throughput quantitative screening of CFTR chloride transport agonists. *Am J Physiol Cell Physiol* **281**, C1734-1742, (2001).
174 De La Fuente, R., Namkung, W., Mills, A. & Verkman, A. S. Small-molecule screen identifies inhibitors of a human intestinal calcium-activated chloride channel. *Mol Pharmacol* **73**, 758-768, (2008).
175 Sui, J. *et al.* Optimization of a Yellow fluorescent protein-based iodide influx high-throughput screening assay for cystic fibrosis transmembrane conductance regulator (CFTR) modulators. *Assay Drug Dev Technol* **8**, 656-668, (2010).
176 Galietta, L. J. *et al.* Novel CFTR chloride channel activators identified by screening of combinatorial libraries based on flavone and benzoquinolizinium lead compounds. *J Biol Chem* **276**, 19723-19728, (2001).
177 Pedemonte, N. *et al.* Phenylglycine and sulfonamide correctors of defective delta F508 and G551D cystic fibrosis transmembrane conductance regulator chloride-channel gating. *Mol Pharmacol* **67**, 1797-1807, (2005).
178 Yang, H. *et al.* Nanomolar affinity small molecule correctors of defective Delta F508-CFTR chloride channel gating. *J Biol Chem* **278**, 35079-35085, (2003).
179 Van Goor, F. *et al.* Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. *Am J Physiol Lung Cell Mol Physiol* **290**, L1117-1130, (2006).
180 Carlile, G. W. *et al.* Correctors of protein trafficking defects identified by a novel high-throughput screening assay. *Chembiochem* **8**, 1012-1020, (2007).
181 Schmidt, A., Mendoza, J. L. & Thomas, P. J. Biochemical and biophysical approaches to probe CFTR structure. *Methods Mol Biol* **741**, 365-376, (2011).
182 Wilke, M. *et al.* Mouse models of cystic fibrosis: phenotypic analysis and research applications. *J Cyst Fibros* **10 Suppl 2**, S152-171, (2011).
183 Keiser, N. W. & Engelhardt, J. F. New animal models of cystic fibrosis: what are they teaching us? *Curr Opin Pulm Med* **17**, 478-483, (2011).
184 Guilbault, C., Saeed, Z., Downey, G. P. & Radzioch, D. Cystic fibrosis mouse models. *Am J Respir Cell Mol Biol* **36**, 1-7, (2007).
185 Sheppard, D. N. *et al.* The patch-clamp and planar lipid bilayer techniques: powerful and versatile tools to investigate the CFTR Cl- channel. *J Cyst Fibros* **3 Suppl 2**, 101-108, (2004).
186 Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-265, (2009).
187 Jung, P. *et al.* Isolation and in vitro expansion of human colonic stem cells. *Nat Med* **17**, 1225-1227, (2011).
188 Dekkers, J. F. *et al.* A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* **19**, 939-945, (2013).
189 Dekkers, J. F., van der Ent, C. K. & Beekman, J. M. Novel opportunities for CFTR-targeting drug development using organoids. *Rare Dis* **1**, e27112, (2013).
190 Dong, Q. *et al.* Human-mouse cystic fibrosis transmembrane conductance regulator (CFTR) chimeras identify regions that partially rescue CFTR-DeltaF508 processing and alter its gating defect. *Proc Natl Acad Sci U S A* **109**, 917-922, (2012).
191 Mall, M., Grubb, B. R., Harkema, J. R., O'Neal, W. K. & Boucher, R. C. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* **10**, 487-493, (2004).
192 Sun, X. *et al.* Adeno-associated virus-targeted disruption of the CFTR gene in cloned ferrets. *J Clin Invest* **118**, 1578-1583, (2008).

193 Rogers, C. S. *et al.* The porcine lung as a potential model for cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* **295**, L240-263, (2008).
194 van Ewijk, B. E., van der Zalm, M. M., Wolfs, T. F. & van der Ent, C. K. Viral respiratory infections in cystic fibrosis. *J Cyst Fibros* **4 Suppl 2**, 31-36, (2005).
195 Brennan, S. Innate immune activation and cystic fibrosis. *Paediatr Respir Rev* **9**, 271-279; quiz 279-280, (2008).
196 Elizur, A., Cannon, C. L. & Ferkol, T. W. Airway inflammation in cystic fibrosis. *Chest* **133**, 489-495, (2008).
197 Courville, C. A. *et al.* Acquired defects in CFTR-dependent beta-adrenergic sweat secretion in chronic obstructive pulmonary disease. *Respir Res* **15**, 25, (2014).
198 Dransfield, M. T. *et al.* Acquired cystic fibrosis transmembrane conductance regulator dysfunction in the lower airways in COPD. *Chest* **144**, 498-506, (2013).
199 Galietta, L. J. *et al.* Modification of transepithelial ion transport in human cultured bronchial epithelial cells by interferon-gamma. *Am J Physiol Lung Cell Mol Physiol* **278**, L1186-1194, (2000).
200 Snodgrass, S. M., Cihil, K. M., Cornuet, P. K., Myerburg, M. M. & Swiatecka-Urban, A. Tgf-beta1 inhibits Cftr biogenesis and prevents functional rescue of DeltaF508-Cftr in primary differentiated human bronchial epithelial cells. *PLoS One* **8**, e63167, (2013).
201 Alton, E. W. *et al.* Repeated nebulisation of non-viral CFTR gene therapy in patients with cystic fibrosis: a randomised, double-blind, placebo-controlled, phase 2b trial. *Lancet Respir Med* **3**, 684-691, (2015).

2

A novel fluorescent sensor for measurement of CFTR function by flow cytometry

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Abstract

Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene cause cystic fibrosis. CFTR-dependent iodide transport measured by fluorescent quenching of ectopically expressed halide-sensitive YFP is widely being used to study CFTR function by microscopy or plate readers. Since YFP fluorescence in these systems is dependent on YFP expression levels and iodide concentration, differences in sensor expression level between experimental units are normalized at the start of each experiment. To allow accurate measurement of CFTR function by flow cytometry, we reasoned that co-expression of an iodide insensitive fluorescent protein would allow for normalization of sensor expression levels and more accurate quantification of CFTR function. Our data indicated that dsRed and mKate fluorescence are iodide-insensitive, and we determined an optimal format for co-expression of these fluorescent proteins with halide-sensitive YFP. We showed using microscopy that ratiometric measurement (YFP/mKate) corrects for differences in sensor expression levels. Ratiometric measurements were essential to accurately measure CFTR function by flow cytometry that we here describe for the first time. Mixing of wild type or mutant CFTR expressing cells indicated that addition of approximately 10% of wild type CFTR expressing cells could be distinguished by ratiometric YFP quenching. Flow cytometric ratiometric YFP quenching also allowed us to study CFTR mutants associated with differential residual function upon ectopic expression. Compared with conventional plate-bound CFTR function assays, the flow cytometric approach described here can be used to study CFTR function in suspension cells. It may be further adapted to study CFTR function in heterologous cell populations using cell surface markers, and selection of cells that display high CFTR function by cell sorting.

Introduction

Cystic fibrosis (CF) is the most common life-shortening recessive disease in the Caucasian population, and results from mutation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene [1]. CF is a multi-organ disease, and disease progression is highly variable between patients. Recent data indicates that CFTR-deficiency in immune cells may further contribute to the heightened inflammatory responses, and increased pathogen pressure [2-3]. However, still little is known on CFTR function in various immune cell subsets, and more methods to analyse CFTR function in such cell types are needed.

The most prevalent loss-of-function CFTR mutation is the deletion of phenylalanine 508 (F508del), but over 1500 mutations have been associated with CF (www.genet.sickkids.on.ca/www.CFTR2.org). These mutations have been classified according to their impact on CFTR function [4]. In general, class I mutations affect CFTR translation (stop codons, e.g. G542X), class II mutations involve the folding and apical trafficking of CFTR (e.g. F508del). Class III (e.g. G551D), IV (e.g. R117H) and V affect CFTR gating, conductance and splicing, respectively. Novel drugs are being developed that target mutation-specific defects of CFTR from which correctors that improve apical trafficking of CFTR such as VX-809 [5] and potentiators that improve CFTR gating, such as VX-770 [6], are highly promising [7].

Multiple techniques have been described to study CFTR function including electrophysiological approaches such as Patch-clamp recordings and short-circuit current measurements in Ussing chambers, or influx and efflux measurements of radioactive ions [8]. From these assays, iodide-mediated quenching rates of an ectopically expressed mutant form of YFP has been widely used as a relative simple assay to measure CFTR function upon introduction or removal of iodide [9-11]. This assay is optimally suited for CFTR function measurement in plate-bound adherent cells, and accurate CFTR function measurement are achieved through normalization for YFP expressing at the start of the experiment.

Here, we developed a ratiometric sensor to measure CFTR function based on the halide-sensitive YFP and a halide-insensitive red fluorescent protein. Our ratiometric indicator effectively corrects for differences in sensor expression level between experimental units such as individual cells, and can accurately measure CFTR-dependent iodide in- and efflux. Our sensor is particularly suited to study CFTR function when variability in sensor expression levels cannot be normalized in a specific experimental setup as we show here for flow cytometry. The method described here may also be used to study CFTR function in suspension cells or yeast cells which are used to express CFTR for crystallization studies [12].

Materials and Methods

Cell culture and plasmids - BHK (Baby Hamster Kidney) cells stably expressing CFTR-WT (BHK-CFTR-WT) or CFTR-F508del (BHK-F508del) [13] were maintained in DMEM (Invitrogen) supplemented with 8% heat-inactivated FCS, penicillin and streptomycin (Invitrogen) and methotrexate at 37°C and 5% CO_2. Venus-constructs were made by overlap extension PCR [14] from Venus constructs previously described [15]. pcDNA3-Venus-linker-dsRed was constructed by PCR-amplification of Venus and dsRed and sequential ligation into pcDNA3 which already contained the linker [15]. Halide sensitive Venus was created by introducing the H148Q/I152L mutations [9] by QuickChange™ (Stratagene, La Jolla, CA) site directed mutagenesis method using Phusion® polymerase (New England Biolabs, Ipswich, MA). pcDNA3-Venus-E2A-dsRed was created by replacing the linker in Venus-linker-dsRed by the autocleavable peptide E2A [16]. pcDNA3-Venus-E2A-mKate was constructed by replacing dsRed by mKate2 [17]. pcDNA3-YFP-E2A-mKate was constructed by replacing Venus for YFP, which is constructed from pEYFP-N1 (Clontech, Mountain View, CA) with the additional H148Q/I152L and F46L [18] mutations for increased iodide sensitivity and fluorescent intensities, respectively. CFTR mutants were made by QuickChange™ site-directed

mutagenesis on pcDNA3-CFTR-WT using Phusion® polymerase. Primers were designed to extent approximately 15 bp up and downstream of the site of mutagenesis, but always ending with C or G. The PCR program used for the CFTR-mutants was as follows: 98°C for 60 seconds; followed by 25 cycles 98°C for 25 seconds, 55°C for 25 seconds and 72°C for 10 minutes; final elongation step was at 72°C for 10 minutes. All constructs were sequence verified. BHK cells were lentivirally transduced using Phage2-YFP-E2A-mKate carrying a puromycin selection cassette. Phage2 backbone was a gift from Dr. D. D'Astolfo (Hubrecht Institute, The Netherlands).

YFP quenching by confocal microscopy - BHK cells stably expressing CFTR and YFP-E2A-mKate were plated two days before their use in confocal experiments. For ectopic expression of Venus/YFP-constructs, BHK cells were plated one day before transfection. Cells were transfected using Polyethylenimine (PEI, Linear (MW 25,000), Polysciences Inc., Warrington, PA) as described [19]. Two days after transfection BHK cells were washed twice with an iodide-lacking buffer (in mM: 137 NaCl, 2.7 KCl, 0.7 $CaCl_2$, 1.1 $MgCl_2$, 1.5 KH_2PO_4, 8.1 Na_2HPO_4). Cells were stimulated with forskolin (25μM) and genistein (50μM) or VX-770 (10μM) for 20 minutes and placed in a buffer containing iodide (similar buffer but NaCl is replaced by equimolars of NaI). At least 3 seconds were recorded to establish a baseline prior the addition of NaI-containing buffer. YFP and dsRed were excited using a 25-40mW argon laser (488nm, at 2.8% power of low intensity setting), mKate was excited using 50 mW diode-pumped solid-state laser (561nm at 0.6% laser power). Pixel dwell time was 1.27μs and two scans were averaged. Changes in fluorescence were monitored by a Zeiss LSM 710 confocal microscope via a spectral detector set to measure from 488nm to 728nm with 9nm intervals; images were recorded every 1.5 seconds.

Data analysis for confocal microscopy - ZEN 2009 (Carl Zeiss MicroImaging GmbH, Jena, Germany) software was used for the separation of YFP and mKate spectra. For YFP the region 493nm to 551nm and for mKate 619nm to 709nm was used. Per condition 20 randomly selected cells were used to calculate the rate of YFP/mKate quenching. The YFP/mKate values were calculated per cell in time and averaged per experimental condition. For rate of iodide influx calculation GraphPad Prism (version 5.03 for Windows) was used to fit a one-phase decay line through the YFP/mKate values. Rate constant K was used as measure of iodide influx.

YFP quenching by flow cytometry - BHK cells were plated one day prior to transfection using PEI as transfection reagent with Venus/YFP-constructs and or CFTR constructs. Two days post transfection the BHK cells were suspended using PBS containing 5mM EDTA and washed twice in a buffer containing or lacking iodide and finally resuspended in the buffer also used for confocal experiments supplemented with forskolin (25μM) and genistein (50μM). After 10 minutes the cells were transferred to a buffer with or without iodide and the changes in fluorescence were monitored in time by a BD FACSCanto II flow cytometer equipped with FACSDiva software for acquisition and data analysis. YFP and dsRed were excited using the 488nm solid state laser (20mW) and mKate was exited using the 633nm HeNe laser (17mW).

Data analysis for flow cytometry - Cells were selected based on forward and side scatter plots and non-transfected cells were excluded based on dsRed or mKate fluorescence, measured in PE (585/42nm) or APC (660/20nm) channels, respectively. YFP fluorescence was recorded in the FITC channel (530/30nm). YFP/dsRed or YFP/mKate ratio's are calculated and monitored using the FACSDiva software. Prior to each influx/efflux measurement each sample was measured to obtain a baseline measurement. For ratiometric measurements data was pooled per 5 seconds and the change in fluorescence over the first 15 seconds was used as measure for CFTR activity.

Western blot analysis - Western blot analysis was performed as described previously [20], L12B4 (Chemicon international, Temecula, CA) was used to detect CFTR. Rabbit polyclonal HSP90 antibody was purchased from Ineke Braakman (Utrecht University, The Netherlands). Neomycin phosphotransferase II was detected by using a rabbit polyclonal antibody (Merck Millipore, Billerica, MA). Primary antibodies were detected using polyclonal rabbit (DAKO, Glostrup, Denmark) or goat horseradish peroxidase-conjugated secondary antibody (Thermo Fisher Scientific Inc., Rockford, IL). Visualization was performed using enhanced chemiluminiscent (GE Healthcare Europe GmbH, Freiburg, Germany), and films (Fuji Medical X-ray film, Tokyo, Japan).

Results

Iodide-sensitive YFP has been used extensively to measure CFTR function. One disadvantage of this system is that YFP fluorescent intensity is both dependent of YFP expression level and intracellular iodide concentrations. We reasoned that co-expression

of a halide-insensitive fluorescent protein from the same expression vehicle would act as internal control, and allow for normalization of sensor expression level during measurements (schematically represented in Fig. 1A). In this approach, the ratio between YFP and another fluorescent protein is assayed upon substitution of a chloride-rich assay buffer for an iodide-rich buffer (influx rate) and subsequently for chloride-rich buffer (efflux rate). To measure CFTR-mediated iodide transport, cells are pre-incubated with specific agonists such as forskolin, which raises cyclic AMP to induce protein kinase A mediated CFTR gating, and genistein, which potentiates CFTR channel activity.

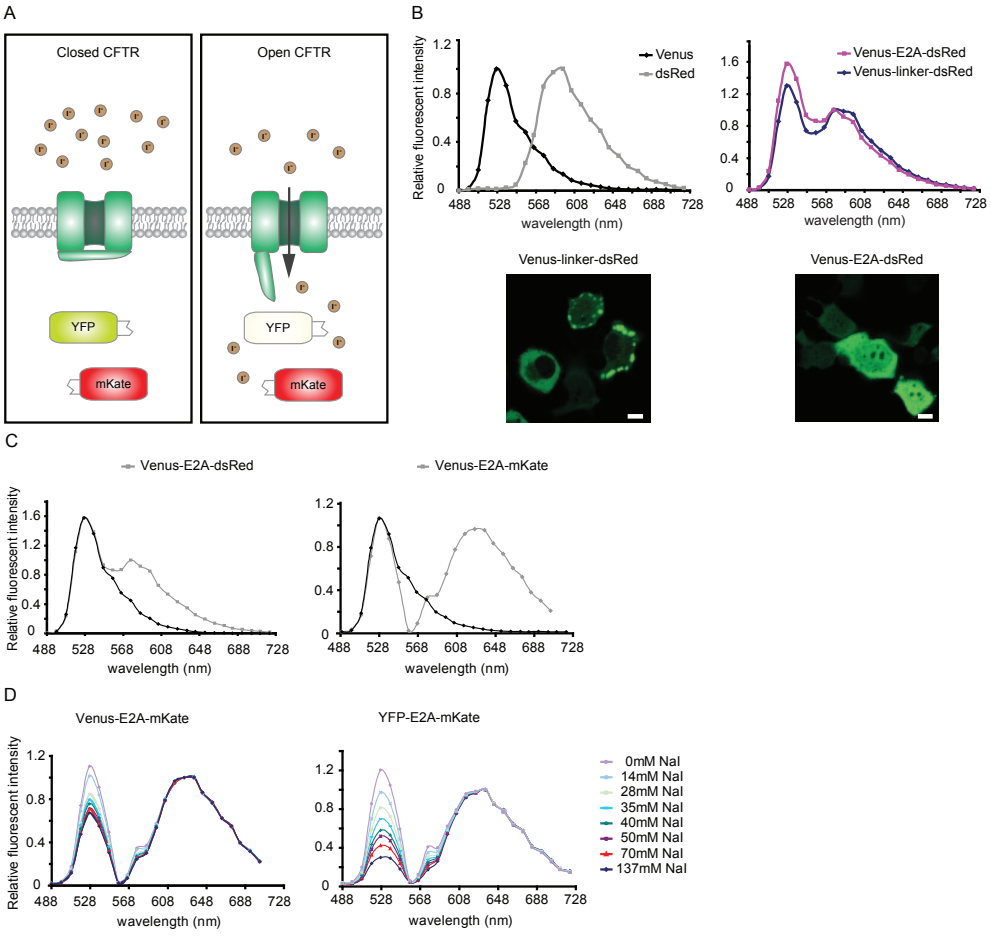

Figure 1. Characterization of iodide-sensitive fluorescent ratiometric sensors by confocal microscopy.
(a) Schematic representation of CFTR-dependent iodide quenching of a ratiometric YFP-based sensor. CFTR channel opening allows diffusion of iodide into the cell leading to specific quenching of YFP but not mKate. (b) Upper panel: fluorescent emission spectra of Venus and dsRed separately expressed in BHK cells (left panel) or coexpressed (right panel). Overlay of Venus-dsRed separated by a polypeptide linker or autocleavable peptide E2A and normalized for dsRed fluorescent intensity (right panel). Lower panels: confocal images of BHK cells transfected with Venus and dsRed, which was physically linked by a polypeptide or the autocleavable peptide E2A. Scale bar: 10 µm. (c) Overlay of emission spectra of Venus (black) and Venus-E2A-dsRed (left panel) or Venus-E2A-mKate (right panel). D. Emission spectra of Venus-E2A-mKate (left panel) and YFP-E2A-mKate (right panel) in BHK-CFTR-WT cells stimulated with forskolin and incubated with different iodide concentrations.

We first selected a correct format for co-expression of two fluorescent proteins. We analyzed fluorescent intensities and subcellular distribution of Venus (a bright version of YFP) and dsRed upon co-expression of these proteins as a linked fusion protein, or as separate entities by replacing the linker peptide in between Venus and dsRed for an auto-cleaving E2A peptide. We observed increased Venus intensities when both proteins were separately expressed upon E2A peptide-mediated cleavage, possibly due to some absorption of Venus fluorescence by dsRed when both proteins are physically linked (Fig. 1B, upper panel). The fusion protein also formed aggregates more frequently upon ectopic expression in cells (Fig. 1B, lower panel). We therefore continued with an E2A cleavable peptide as optimal expression format.

The fluorescent emission spectra of Venus and dsRed when both excited at 488nm are somewhat overlapping which complicates their individual measurement in confocal microscopy (Fig. 1B, upper panel). Therefore, we replaced dsRed for mKate, a bright far red fluorescent protein that is optimally excited at 561nm and displays a large stokes shift (Fig. 1C). We engineered halide sensitive versions of Venus and YFP by introducing H148Q and I152L mutations as previously described [9]. We initially compared the fluorescent spectra of halide-sensitive Venus-E2A-mKate and YFP-E2A-mKate upon incubation of cells with increasing iodide concentrations in wild-type (WT) CFTR-expressing cells in the presence of CFTR activators (Fig. 1D). As expected, we observed that mKate fluorescence is unaffected by iodide concentration, but that YFP is sensitive to iodide. As was previously indicated we also observed that YFP was more sensitive to iodide than Venus [18]. It is important to indicate that the apparent clear distinction between the YFP and mKate signals results from our experimental setup. We simultaneously analyze the entire emission spectra of the cells during our measurement, but emitted light is specifically blocked at the excitation wavelength (561nm) for mKate. The small iodide-sensitive peak around 575nm thus results from YFP. Together, we concluded that intracellular iodide could be most optimally measured by confocal microscopy using a dual-wavelength ratiometric sensor consisting of YFP and mKate separated by a cleavable peptide.

To measure CFTR dependent iodide influx, BHK cells expressing CFTR-WT (BHK-CFTR) or CFTR-F508del (BHK-F508del) were lentivirally transduced with YFP-E2A-mKate to generate stable sensor-positive lines. BHK-CFTR cells were stimulated with forskolin and the CFTR potentiator genistein for 20 minutes in a chloride-containing buffer. Upon replacement of chloride for iodide, we observed a rapid decline in YFP/mKate ratio in time (Fig. 2A). We quantified these responses by measurement of the specific YFP and mKate signals in our emission spectrum (Suppl. Fig. 1A). Forskolin in combination with genistein both stimulated rapid influx of iodide in BHK-CFTR-WT cells (Fig. 2B; Suppl. Fig. 1B shows responses using VX-770 to potentiate CFTR). We next assessed whether CFTR-F508del correction could be measured using our fluorescent sensor. Upon buffer exchange, no significant iodide influx was observed when BHK-F508del cells were unstimulated, but upon stimulation with forskolin and genistein YFP/mKate ratios clearly decreased, which was more prominent when cells were pre-incubated with the CFTR corrector VX-809 (Fig. 2C+D, Suppl. Fig. 1C indicates the individual responses of each experiment). Iodide mediated YFP quenching in CFTR-F508del expressing cells was observed using when using forskolin and VX-770, which was stronger upon preincubation with VX-809 (Suppl. Fig. 1D+E). We observed a linear relation between the YFP and mKate fluorescent intensities indicating that expression of both proteins was similar, independent of sensor expression level (Suppl. Fig. 2A+B). We also did not find a

correlation between YFP/mKate ratio before iodide addition and YFP or mKate expression (Suppl. Fig 2C). Furthermore we found no correlation between the YFP quenching rate and the initial YFP/mKate ratio (Suppl. Fig. 2D). Together, these data indicated that wild-type and mutant CFTR function can be assessed using a dual wavelength ratiometric sensor by confocal microscopy.

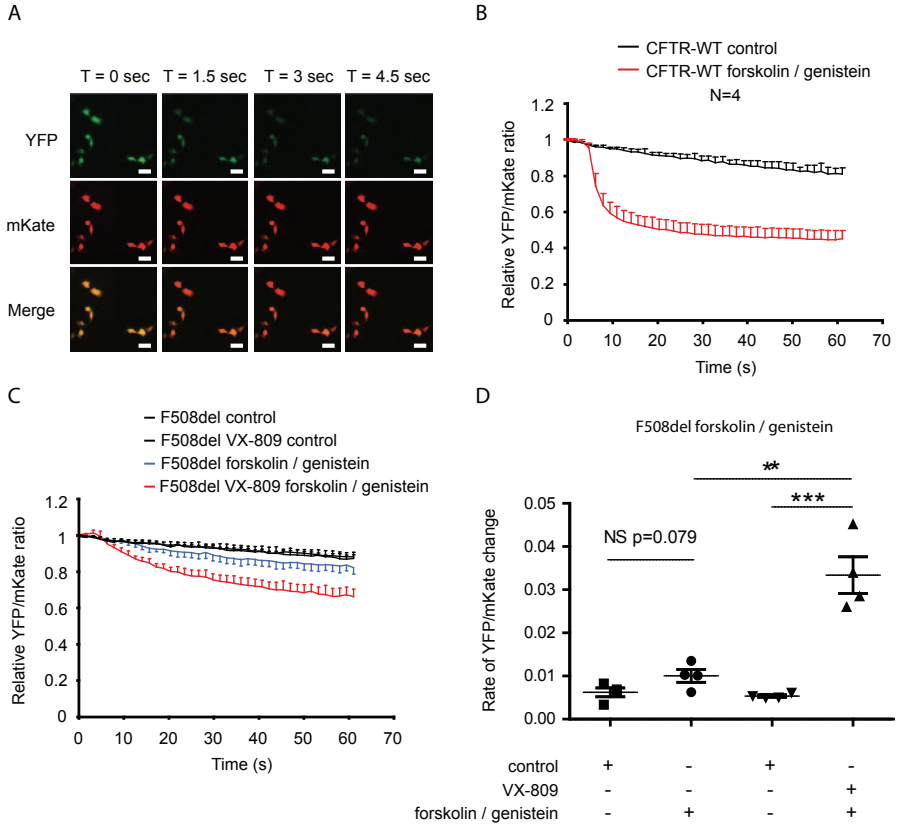

Figure 2. Measurement of CFTR function using a ratiometric sensor by confocal microscopy.
(a) Representative examples of forskolin-stimulated BHK-CFTR-WT cells expressing YFP-E2A-mKate upon addition of iodide in time. Scale bar: 50 µm. (b) BHK-CFTR-WT cells stably expressing YFP-E2A-mKate were preincubated with forskolin and genistein (red) or vehicle (black) for 20 min (average of four independent experiments ± SEM). Baseline values were established for 3 s before addition of iodide. (c) BHK-F508del cells stably expressing YFP-E2A-mKate were stimulated for 20 min with forskolin and genistein in the absence (blue) or presence of VX-809 (red, preincubated for 24 h). Solvent control is indicated in black. Data are averaged of four independent experiments ± SEM. (d) Quantification of YFP/mKate quenching rate of four independent experiments as described under Figure 2C. Data are mean ± SEM. ***P < 0.001, **P < 0.01, NS: not significant.

In standard halide-quenching assays, differences in YFP expression between the experimental units are normalized by measurement of baseline YFP values before addition of iodide. Using ratiometric measurement, normalization for YFP expression level can be performed during the experiment that can lead to improved accuracy of CFTR function measurement depending on the experimental set up. Within our data set of VX-809 treated BHK-F508del cells stimulated with forskolin and genistein, we compared CFTR-dependent

influx rates as measured by the decline in YFP levels or decline in YFP/mKate ratio, either with or without normalization for baseline values. As expected, the average decline in YFP levels and YFP/mKate levels were comparable, independent of normalization for baseline values (Fig. 3). Normalization for expression levels either by baseline correction (F/F_0), or by ratiometry reduced the standard deviations of our measurement to similar levels. However, we did observed a slight reduction of 4% of standard deviations in our measurements when ratiometric data was also normalized for baseline when compared to baseline-corrected YFP measurements (n=5, $p<0.05$). This indicates that ratiometric measurement corrects for variability in sensor expression level, and can, to a minor extent, improve accuracy when data is expressed relative to values before addition of iodide.

Our previous data suggests that ratiometric measurement is particularly suited to study CFTR function when sensor expression levels cannot be normalized for baseline values. We therefore assessed whether our ratiometric sensor would be suited to study CFTR function using flow cytometry, which is a preferred system for analysis of CFTR function in suspension cells, and allows for easy separation and quantification of the fluorescent signals. However, as each cell is lost upon measurement, correction for baseline sensor levels is more difficult.

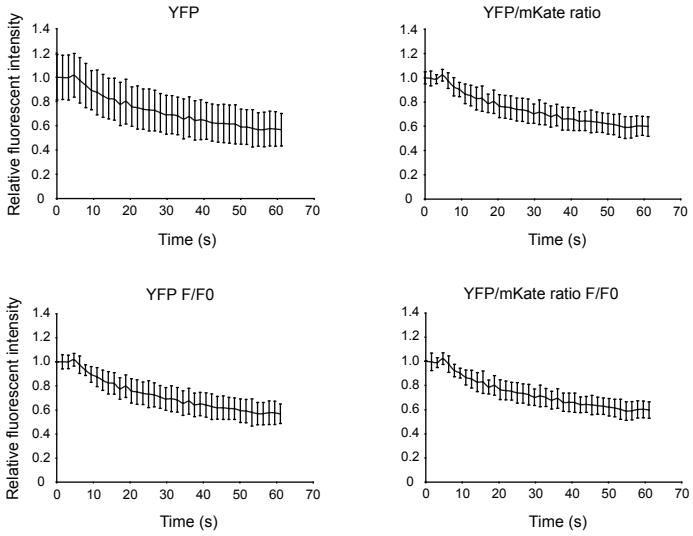

Figure 3. Comparison of ratiometric and YFP data sets of BHK-F508del cells stably expressing YFP-E2A-mKate. Iodide influx in F508del expressing BHK cells treated with VX-809 and stimulated with forskolin and genistein. Average iodide influx traces of 20 individual cells analyzed for only YFP quenching; YFP/mKate quenching and after correction for fluorescence per cell (F/F0) before the addition of iodide for YFP and YFP/mKate. Data are mean ± SD. One representative example from four independent experiments is indicated.

Using flow cytometry, we analyzed iodide-responsiveness of our different sensors upon transfection in BHK cells (Fig. 4A). Again, the YFP-E2A-mKate was most sensitive to iodide, but Venus co-expressed with either mKate or dsRed demonstrated a more linear dependency on iodide compared to YFP. Hereafter, we measured the iodide influx rates in BHK-CFTR cells ectopically expressing the Venus-E2A-mKate. We selected cells based on FSC

and SSC and expression of mKate (30 to 50% of transfection efficiency) and analysed the Venus/mKate ratio in time upon addition of an iodide rich buffer during online measurement (Fig. 4B). For BHK-CFTR-WT cells we observed a rapid iodide influx that was absent in cells expressing F508del (Fig. 4C). The highly sensitive YFP-sensor was not optimally suited for flow cytometry due to rapid quenching of YFP after addition of an iodide rich buffer to the cells, and the lag time of approximately 5 seconds before measurements could be started (Suppl. Fig 3). Therefore all following experiments were performed using the Venus-based fluorescent sensor.

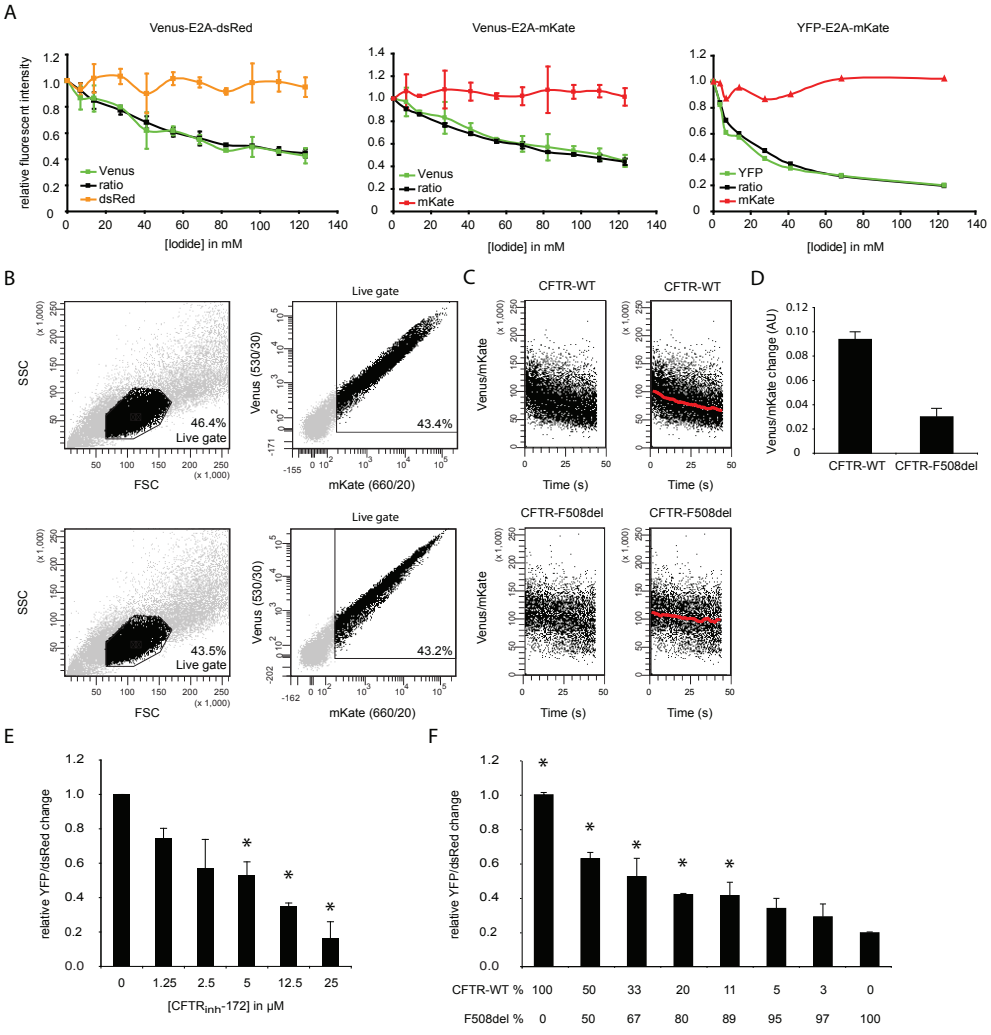

Figure 4. CFTR measurement by ratiometric YFP quenching using flow cytometry.
(a) BHK-CFTR-WT cells transiently expressing indicated ratiometric sensors were stimulated with forskolin and genistein (10 min) in the presence of different iodide concentrations. Filters used for Venus and YFP: 530/30 nm, dsRed: 585/42 nm, mKate: 660/20 nm. (b) BHK cells transiently expressing CFTR-WT (top) or CFTR-F508del (bottom) and Venus-E2A-mKate were selected based on forward and side scatter (left panel) and sensor expression, mKate positive cells (30—50% of the cells in the FSC and SSC gate, middle panel). (c) Cells were stimulated with forskolin

and genistein and fluorescent intensities measured in time after dilution in an iodide-containing buffer. Red line indicates the mean intensity of Venus/mKate (right panel). (d) Quantification of Venus/mKate change by calculating the decline of YFP/mKate over the first 15 s. Data are mean ± SEM (n = 4). (e) BHK-CFTR-WT cells were preincubated for 10 min in the presence of forskolin, genistein, and $CFTR_{inh}$-172 at the indicated concentrations prior the addition of iodide (*P < 0.05 compared with control). (f) BHK cells expressing CFTR-WT or CFTR-F508del were mixed in different ratios, stimulated for 10 min in the presence of forskolin and genistein prior the addition of iodide (right panel). Data are mean ± SD, of two independent experiments (*P < 0.05 compared with BHK-F508del only).

We further validated CFTR dependency of YFP-quenching in this approach by preincubation with the specific CFTR inhibitor $CFTR_{inh}$-172. We observed that iodide mediated YFP quenching was inhibited by $CFTR_{inh}$-172 in a dose dependent manner (Fig. 4D). To assess the sensitivity of this assay, BHK cells stably expressing CFTR-WT or -F508del were mixed in different ratios and YFP quenching was monitored in time (Fig. 4E). We could clearly distinguish 10% of wt cells in F508del background using this method. These data demonstrated the ability of our novel fluorescent sensor to measure CFTR activity by flow cytometry.

We next assessed iodide influx / efflux rates of BHK cells expressing different CFTR-mutations belonging to different classes of mutations that are associated with differences in residual function. We co-transfected the fluorescent sensor with different CFTR-constructs in a 1:10 ratio to ensure that cells transfected with the fluorescent sensor were also transfected with CFTR. Gating strategy was similar as shown in Figure 4B. As expected cells transfected with CFTR-WT showed a rapid iodide influx and efflux, whereas CFTR-F508del responded at rates comparable to empty vector transfected BHK cells. CFTR-G551D showed an intermediate response after stimulation with forskolin and genistein. Interestingly the CFTR-R117H displayed iodide influx rates similar to CFTR-WT, whereas the efflux rates were smaller than for CFTR-WT (Fig. 5A, left panel). From these data, we also determined CFTR function by analysis of YFP data alone (Fig. 5A, right panel). As expected larger variation was present in this data set preventing accurate measurement on CFTR function. This shows the importance of the ratiometric measurements when cells (or experimental units) cannot be monitored in time.

As controls, Western blots were prepared to validate expression of our constructs (Fig. 5C). YFP-quenching was only observed in BHK cells expressing CFTR-WT or CFTR-mutant proteins which also showed expression of the complex-glycosylated CFTR C-band, which is associated with apical membrane expression of CFTR and residual function [21]. F508del is not properly folded and retained within the ER membrane causing expressing of solely the core-glycosylated CFTR B-band that is rapidly degraded. The G542X mutation contains a premature termination codon which is located before the glycosylation site and migrates as a single band at a lower molecular weight. HSP90 expression was used to confirm equal loading. Neomycin phosphotransferase II that is expressed from the same expression vector as CFTR was used as transfection control. In conclusion these data show that ratiometric measurements are essential to accurately measure CFTR function by YFP-quenching using flow cytometry.

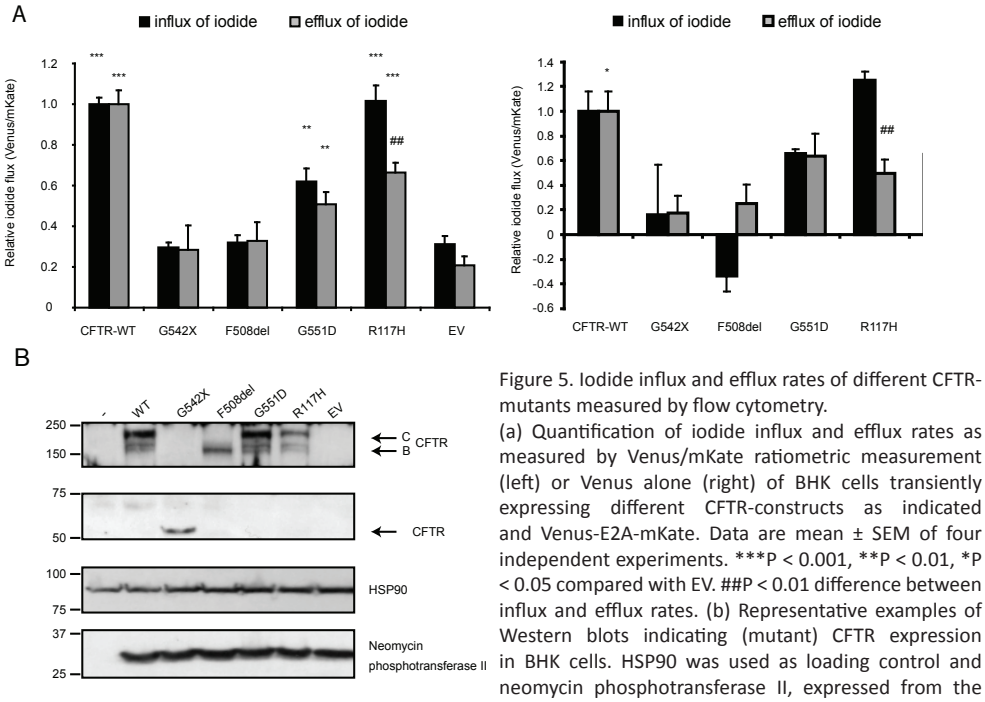

Figure 5. Iodide influx and efflux rates of different CFTR-mutants measured by flow cytometry.
(a) Quantification of iodide influx and efflux rates as measured by Venus/mKate ratiometric measurement (left) or Venus alone (right) of BHK cells transiently expressing different CFTR-constructs as indicated and Venus-E2A-mKate. Data are mean ± SEM of four independent experiments. ***P < 0.001, **P < 0.01, *P < 0.05 compared with EV. ##P < 0.01 difference between influx and efflux rates. (b) Representative examples of Western blots indicating (mutant) CFTR expression in BHK cells. HSP90 was used as loading control and neomycin phosphotransferase II, expressed from the same vector as CFTR, was used as transfection control. First lane is untransfected BHK cells.

Discussion

The purpose of this study was to develop a novel approach to measure CFTR function by flow cytometry. Therefore we adapted a halide-sensitive YFP quenching assay in which we co-expressed a non-halide sensitive fluorescent protein to correct for sensor expression level. Here, we used it to study CFTR-mediated iodide transport, and found similar properties for our sensors and the previously published YFP-based sensors. This novel ratiometric sensor is ideally suited to study CFTR function when sensor expression levels cannot be corrected for at the level of individual experimental units.

We described two critical properties of the ratiometric sensor for optimal measurement of iodide transport. First, physical attachment of two fluorescent proteins can lower the sensitivity of the YFP probe as we observed higher YFP fluorescent levels for YFP-E2A-dsRed than for YFP-linker-dsRed. This is likely due to Förster Resonance Energy Transfer (FRET) between YFP and dsRed when the molecules are in close proximity due to physical linkage. The potential drawback of separating the two fluorescent proteins is that their levels may change upon expression due to differences in turnover rates. However, since we our measurements are short, it is unlikely that the ratio between YFP and mKate is altered during the time of the experiment, and we therefore favour to separate the proteins due to increased intensity of YFP.

Second, it is critical that the fluorescent signal that is used for normalization is completely insensitive to iodide, and thus also fully separated from YFP-derived signals.

Otherwise, the ratiometric value would loose sensitivity compared to the YFP signal alone. We found increased difficulty to separate the YFP from dsRed as compared to mKate using confocal microscopy, and propose that the latter would be optimal for confocal microscopy due to the possibility to perform dual wavelength excitation, and the larger differences in emission spectra. For flow cytometry, optimal compensation is required to separate YFP from dsRed signals, but this can be easily accomplished.

Our sensor is especially suited to measure CFTR dependent iodide influx when correction for sensor expression level cannot be performed, as we showed in a newly developed flow cytometric approach to analyse CFTR function. Our flow cytometric approach allowed us to quickly investigate CFTR dependent influx or efflux rates in transient expression experiments, and relied on ratiometric normalization for accurate measurement. Compared with confocal microscopy, emission spectra were easier to separate, and fluorescent intensities were easily quantified. The major pitfall is a lag time in fluorescent measurement when the chloride buffer is changed for iodide buffer that results in less clearly defined influx rates that probably leads to underestimation of cells that display high CFTR activity. This can be compensated by using a less iodide-sensitive sensor as shown here, or by using a setup in which the iodide rich buffers can be added directly during on-line measurements. This may explain the relative high activity of CFTR-G551D (impaired gating) and R117H (impaired conductivity) that we observed. For CFTR-R117H, however, it has previously been indicated to mediate currents comparable with CFTR-WT [22]. Intriguingly, our data would suggest that iodide import through CFTR-R117H is quicker as compared to iodide export through R117H.

By studying YFP quenching rates of individual cells that displayed high or low sensor expression levels, or high or low YFP/mKate ratios, we could validate that differences in sensor expression levels, or quenching status before iodide addition do not impact iodide-induced CFTR-mediated quenching. By Flow cytometry, we could also select high and low sensor-expressing cells and found identical CFTR-dependent quenching rates in cells stably expressing CFTR-WT. These data further validated the reliability of CFTR function measurements using genetic YFP-based sensors.

Ratiometric values correct for differences in sensor expression level between expression units in a specific experimental setup during the experiment and thereby reduce experimental variability and improve accuracy. Other dyes such as SPQ (6-methoxy-N-(3-sulfopropyl)quinolinium) [23-24], LZQ (7-(beta-D-ribofuranosylamino)-pyrido[2,1-h]-pteridin-11-ium-5-olate) [25], and DIBAC4(3) (bis-(1,3-dibutylbarbituic acid)trimethine oxonol) [26-27] have been used to study CFTR function using plate-bound assays. Both SPQ and LZQ can be quenched by iodide like YFP, whereas DIBAC4(3) is used to measure membrane potential which changes upon activation of ion channels. A big advantage of these dyes over genetic sensors is that these can be easily added to cells to allow measurements. As loading and leakage efficacy between individual cells cannot be controlled using these single dyes, we expect that genetic sensors like our YFP/mKate sensor to be more ideal to detect small changes at low CFTR activity conditions using flow cytometry.

Ratiometric measurements to quantify intracellular chloride have been previously used. This sensor called Clomeleon specifically uses FRET to measure chloride concentration [28-29]. For flow cytometry, this sensor appears less suited as it depends on a 456 nm laser that is not present on most flow cytometers, although this approach may also be adapted for flow cytometry using a different FRET-donor.

In conclusion, we designed a novel fluorescent sensor to measure CFTR function that allows for correction of expression levels between experimental units based on the already published halide sensitive YFP. Compared with the known YFP sensor it is especially suited for applications in which expression cannot be normalized as we show in a newly developed YFP quenching assay using flow cytometry. Our data indicate that our flow cytometric assay can be an important tool for analysis of CFTR function in suspension cells such as immune cells or yeast cells that can be used to purify functional CFTR protein [12,30]. In addition, our flow cytometric approach may potentially be used to sort cells with high CFTR function from heterogeneous cell mixtures, or assess CFTR function in various cell types simultaneously by including cell surface markers.

Acknowledgements

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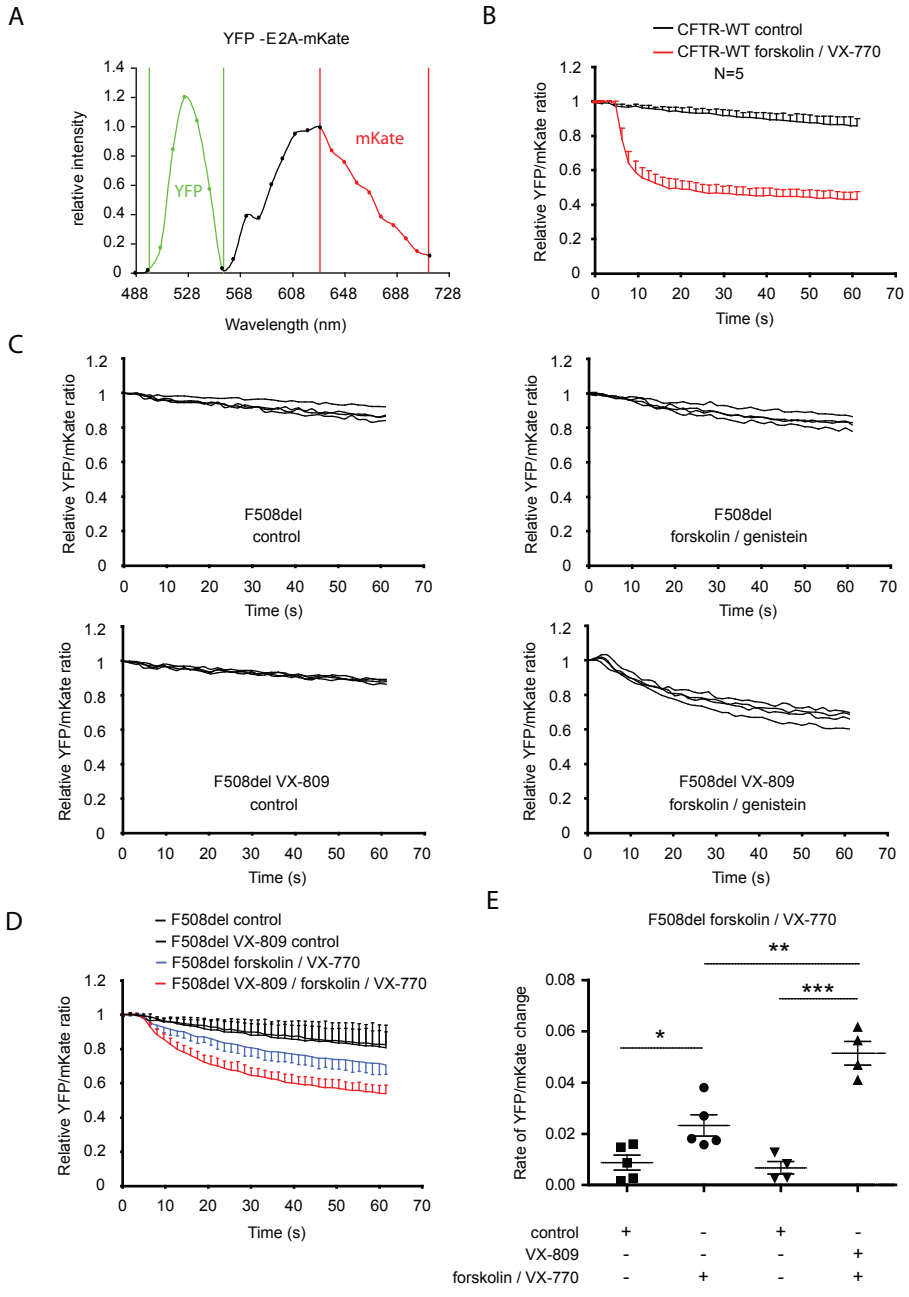
Reference list

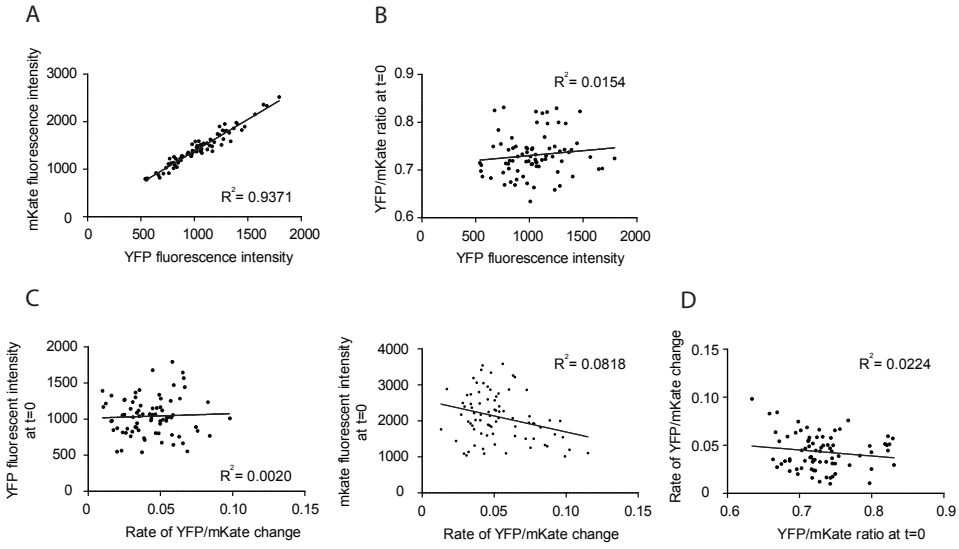
1 Riordan, J. R. CFTR function and prospects for therapy. Annu Rev Biochem 77, 701-726, (2008).
2 Di, A. et al. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. Nat Cell Biol 8, 933-944, (2006).
3 Mueller, C. et al. Lack of cystic fibrosis transmembrane conductance regulator in CD3+ lymphocytes leads to aberrant cytokine secretion and hyperinflammatory adaptive immune responses. Am J Respir Cell Mol Biol 44, 922-929, (2011).
4 Zielenski, J. Genotype and phenotype in cystic fibrosis. Respiration 67, 117-133, (2000).
5 Van Goor, F. et al. Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. Proc Natl Acad Sci U S A 108, 18843-18848, (2011).
6 Van Goor, F. et al. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. Proc Natl Acad Sci U S A 106, 18825-18830, (2009).
7 Ramsey, B. W. et al. A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. N Engl J Med 365, 1663-1672, (2011).
8 Moran, O. & Zegarra-Moran, O. On the measurement of the functional properties of the CFTR. J Cyst Fibros 7, 483-494, (2008).
9 Galietta, L. J., Haggie, P. M. & Verkman, A. S. Green fluorescent protein-based halide indicators with improved chloride and iodide affinities. FEBS Lett 499, 220-224, (2001).
10 Jayaraman, S., Haggie, P., Wachter, R. M., Remington, S. J. & Verkman, A. S. Mechanism and cellular applications of a green fluorescent protein-based halide sensor. J Biol Chem 275, 6047-6050, (2000).
11 Verkman, A. S. & Galietta, L. J. Chloride channels as drug targets. Nat Rev Drug Discov 8, 153-171, (2009).
12 O'Ryan, L., Rimington, T., Cant, N. & Ford, R. C. Expression and purification of the cystic fibrosis transmembrane conductance regulator protein in Saccharomyces cerevisiae. J Vis Exp, (2012).
13 Lukacs, G. L. et al. Conformational maturation of CFTR but not its mutant counterpart (delta F508) occurs in the endoplasmic reticulum and requires ATP. EMBO J 13, 6076-6086, (1994).
14 Beekman, J. M. et al. Modulation of FcgammaRI (CD64) ligand binding by blocking peptides of periplakin. J Biol Chem 279, 33875-33881, (2004).
15 Beekman, J. M., Verhagen, L. P., Geijsen, N. & Coffer, P. J. Regulation of myelopoiesis through syntenin-mediated modulation of IL-5 receptor output. Blood 114, 3917-3927, (2009).
16 Szymczak, A. L. et al. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol 22, 589-594, (2004).
17 Shcherbo, D. et al. Far-red fluorescent tags for protein imaging in living tissues. Biochem J 418, 567-574, (2009).

18 Nagai, T. et al. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol 20, 87-90, (2002).19 Beekman, J. M. et al. Syntenin-mediated regulation of Sox4 proteasomal degradation modulates transcriptional output. Oncogene 31, 2668-2679, (2012).
20 van Meegen, M. A., Terheggen-Lagro, S. W., van der Ent, C. K. & Beekman, J. M. CFTR expression analysis in human nasal epithelial cells by flow cytometry. PLoS One 6, e27658, (2011).
21 Gregory, R. J. et al. Expression and characterization of the cystic fibrosis transmembrane conductance regulator. Nature 347, 382-386, (1990).
22 de Nooijer, R. A. et al. Assessment of CFTR function in homozygous R117H-7T subjects. J Cyst Fibros 10, 326-332, (2011).
23 Lee, R. J. & Foskett, J. K. cAMP-activated Ca2+ signaling is required for CFTR-mediated serous cell fluid secretion in porcine and human airways. J Clin Invest 120, 3137-3148, (2010).
24 Rich, D. P. et al. Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. Nature 347, 358-363, (1990).
25 Jayaraman, S., Teitler, L., Skalski, B. & Verkman, A. S. Long-wavelength iodide-sensitive fluorescent indicators for measurement of functional CFTR expression in cells. Am J Physiol 277, C1008-1018, (1999).
26 Gonzalez, J. E., Oades, K., Leychkis, Y., Harootunian, A. & Negulescu, P. A. Cell-based assays and instrumentation for screening ion-channel targets. Drug Discov Today 4, 431-439, (1999).
27 Van Goor, F. et al. Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. Am J Physiol Lung Cell Mol Physiol 290, L1117-1130, (2006).
28 Kuner, T. & Augustine, G. J. A genetically encoded ratiometric indicator for chloride: capturing chloride transients in cultured hippocampal neurons. Neuron 27, 447-459, (2000).
29 Markova, O., Mukhtarov, M., Real, E., Jacob, Y. & Bregestovski, P. Genetically encoded chloride indicator with improved sensitivity. J Neurosci Methods 170, 67-76, (2008).
30 Huang, P., Stroffekova, K., Cuppoletti, J., Mahanty, S. K. & Scarborough, G. A. Functional expression of the cystic fibrosis transmembrane conductance regulator in yeast. Biochim Biophys Acta 1281, 80-90, (1996).

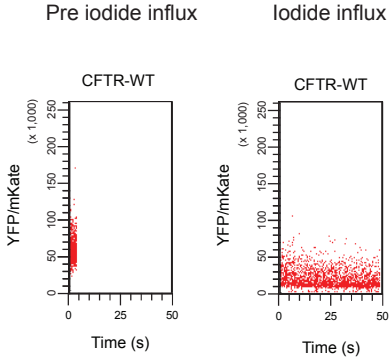


Supplementary Figure S1. Measurement of CFTR function using a ratiometric sensor by confocal microscopy.
(a) Emission spectrum of YFP-E2A-mKate indicating the regions used to quantify YFP (green, 493-551 nm) and mKate (red, 619-709 nm). (b) Forskolin-induced iodide quenching in BHK-CFTR-WT cells. BHK-CFTR-WT cells stably expressing YFP-E2A-mKate were preincubated with forskolin (25µM) and VX-770 (10µM) (red) or vehicle (black) for 20 min. Baseline values were established for 3 s before addition of iodide. The data represents an average of 5 independent experiments (± SEM). (c) Individual data sets for forskolin (25 µM) / genistein (50 µM) (left panels)

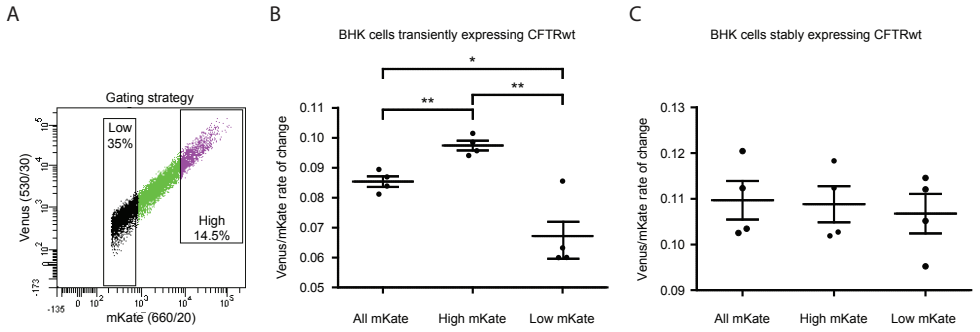
or vehicle (right panels) stimulated BHK-CFTR-F508del cells. 10 µM VX-809 was preincubated for 24 h (Fig. 2C). (d) BHK-F508del cells stably expressing YFP-E2A-mKate were stimulated for 20 min with forskolin (25 µM) and VX-770 (10 µM) in the absence (blue) or presence of VX-809 (10 µM) (red). VX-809 was preincubated for 24 h. Each line represents an average of 4 or 5 independent experiments. (e) Quantification of YFP/mKate quenching rate was calculated by fitting a one-phase decay line through the YFP/mKate values using GraphPad Prism. Rate constant K is used as measure of iodide influx. Data of 4 or 5 independent experiments, presented as mean ± SEM.



Supplementary Figure S2. Correlation analysis between different fluorescent parameters measured by confocal microscopy.
BHK-F508del cells expressing YFP-E2A-mKate were treated for 24 h with VX-809 (10 µM) and stimulated with forskolin (25 µM) and genistein (50 µM) for 20 min. Each point represents one cell. (a) Correlation of YFP and mKate fluorescent intensities prior to the addition of iodide indicates a fixed ratio of YFP and mKate fluorescence independent of sensor expression level. (b) Similar as (a): absence of correlation between YFP fluorescence and YFP/mKate ratio at the start of experiments indicates that the YFP/mKate ratio is independent of expression level of the sensor. (c) The absence of a correlation between the rate of YFP/mKate quenching and YFP or mKate fluorescence indicates that the rate of YFP quenching is independent of sensor expression level. (d) No correlation was found between the rate of YFP/mKate quenching and the initial YFP/mKate ratio measured prior to the addition of iodide. Differences in initial YFP/mKate ratios cannot predict the cellular response to iodide.



Supplementary Figure S3. Iodide mediated quenching in BHK-CFTR-WT cells expressing YFP-E2A-mKate measured by flow cytometry.
BHK cells transiently expressing CFTR-WT and YFP-E2A-mKate, were stimulated for 10 min with Forskolin (25 µM) and genistein (50 µM). Left panel, cells suspended in stimulation buffer lacking iodide. Right panel, after transfer to an iodide containing buffer. YFP is quenched almost completely before the first cells can pass the detector, the lag time is approximately 5 s.



Supplementary Figure S4. Iodide quenching rates in high and low sensor expressing cells by flow cytometry.
(a) Cells expressing different levels of Venus/mKate were gated based on mKate intensity, three groups were selected: all transfected cells, low mKate levels, or high mKate levels. (b) When cells were transiently co-transfected with sensor and CFTR-WT (1:10 ratio), quenching of Venus was higher in cells transfected with high sensor levels. These cells are also expected to have increased levels of co-transfected CFTR. (c) No differences in Venus quenching were observed when high and low sensor-expressing cells were selected upon transient transfection of the sensor in cells stably expressing CFTR-WT. This further indicates that CFTR function measurement by flow cytometry is largely independent of sensor expression levels.

3

Limited premature termination codon suppression by read-through agents in cystic fibrosis intestinal organoids

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Abstract

Premature termination codon read-through drugs offer opportunities for treatment of multiple rare genetic diseases including cystic fibrosis. We here analyzed the read-through efficacy of PTC124 and G418 using human cystic fibrosis intestinal organoids (*E60X/4015delATTT*, *E60X/F508del*, *G542X/F508del*, *R1162X/F508del*, *W1282X/F508del* and *F508del/F508del*). G418-mediated read-through induced only limited CFTR function, but functional restoration of CFTR by PTC124 could not be confirmed. These studies suggest that better read-through agents are needed for robust treatment of nonsense mutations in cystic fibrosis.

Introduction

Cystic fibrosis (CF) is caused by mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene that encodes a cAMP-regulated anion channel [1]. *CFTR* mutations associate with various functional defects and disease severities [2]. CFTR-targeting drugs have been developed that improve *CFTR* mutation-specific defects, such as gating by VX-770 [3] and plasma membrane trafficking by VX-809 [4]. These drugs are not effective as stand-alone treatment for nonsense mutations that are present in approximately 12% of CF subjects and lead to prematurely truncated, non-functional CFTR protein [5].

Aminoglycosides, like G418, have been found to induce read-through of premature termination codons (PTCs) [6], by interacting with the ribosome during translation, and attracting a near cognate aminoacyl-tRNA to the ribosomal A site, to continue translation [7]. To bypass cell toxicity associated with aminoglycosides, PTC124 (Ataluren) was developed as a non-toxic suppressor of premature termination codons [8]. Normal stop codons were not affected, although the mechanism for selectivity of PTC remains unclear [7-8]. PTC124 activity for *CFTR* nonsense mutations has been demonstrated in vitro [9-10], but these data contrast with other studies that fail to demonstrate PTC124 read-through capacity [11-13] . Clinical trials utilizing PTC124 have met inconsistent results, for cystic fibrosis as well as for nonsense-mutated Duchenne muscular dystrophy (DMD) [14-19]. A recent phase III clinical trial in CF did not find significant differences between PTC124 treatment and placebo, but retrospective data analysis suggested that aminoglycoside co-treatment prevented PTC124 activity [16]. Collectively, these data indicate that the read-through activity of PTC124 remains unclear.

Here, we used a functional CFTR assay using human intestinal organoids to measure pharmacological induction of PTC read-through [20]. Organoids are 3D spherical structures that consist of a polarized epithelial cell monolayer surrounding a single lumen, and are formed when adult stem cells from intestinal crypts are cultured in a culture matrix with appropriate growth media [21]. Forskolin induces CFTR-dependent fluid secretion into the organoid lumen, causing rapid swelling of organoids [20]. Here, we investigated organoids with various *CFTR* genotypes to study G418 and PTC124-mediated PTC read-through, and interactions with CFTR-targeting drugs.

Materials and methods

Collection of human organoids - Organoids were generated after intestinal current measurements in human rectal biopsies, obtained for diagnostic care. Informed consent was obtained according to the principles of the declaration of Helsinki.

Human organoid culture and functional CFTR measurements - Crypts were isolated from rectal biopsies of six subjects with cystic fibrosis (*E60X/4015delATTT*, *E60X/F508del*, *G542X/F508del*, *R1162X/F508del*, *W1282X/F508del* and *F508del/F508del*) as previously described [20]. Briefly, after isolation, organoids were cultured for at least 3 weeks prior to CFTR function measurements by forskolin-induced swelling [20]. We chose two concentrations of G418 and PTC124 via dosage titrations (data not shown) and as described in literature, respectively [8]. Organoids were pre-incubated with G418 (100 and 200 μM) PTC124 (1 and 5 μM) and/or VX-809 (3 μM) 24h prior to forskolin stimulation, whereas VX-770 (1 μM) was added simultaneously with forskolin (5 μM). Luminal expansion and organoid swelling were monitored for 60 minutes using a Zeiss LSM 710 confocal microscope. In addition to organoid swelling, forskolin-induced luminal area at t=60 minutes was analyzed blinded as percentage of total organoid surface area per well. Ratio paired t-tests were performed for statistical analysis.

Results

To investigate CFTR repair by PTC124 and G418, we measured swelling of human intestinal organoids compound heterozygous for a *CFTR* PTC allele and a frame shift allele (*E60X/4015delATTT*), lacking CFTR function as indicated by absence of forskolin-induced swelling (FIS) [20]. G418 pretreatment induced very limited FIS indicating CFTR function, but no FIS was detected upon incubation with PTC124. Co-incubation of G418 with CFTR-protein restoring drugs (VX-770+VX-809) enhanced FIS. (Fig. 1A) Induced swelling was very limited, therefore we analyzed the forskolin-induced luminal area (at t=60 minutes, as percentage of total organoid area) to interpret the limited but differential swelling of the single compounds (Fig. 1B, C). We observed luminal area expansion by G418, whereas no effect was measured using PTC124 (Fig. 1C, D). G418 treatment did not increase luminal swelling of *F508del* homozygous organoids, demonstrating PTC specificity (Fig. 1C). These data confirmed the read-through activity of G418 but not of PTC124.

We next assessed read-through by G418 and PTC124 in organoids compound heterozygous for *F508del* and a nonsense mutation (*E60X*, *G542X*, *R1162X* and *W1282X*) (Fig. 2A). Subject genotype *R1162X/F508del* is a low responder, indicated by standard swelling assays performed in our laboratory (data not shown). G418 induced limited read-through in PTC-mutant organoids, which was significant when data from the four different patients was pooled, compared to non-treated organoids (Fig. 2B). Functional restoration was not observed using PTC124 for any of the donor organoids. Targeting the single CFTR-F508del allele by combined VX-770 and VX-809 treatment was most effective in all organoids. These data confirmed the read-through capacity of G418 for different PTCs, but the efficacy is limited compared to F508del-targeting drugs. (Fig. 2)

Discussion

We investigated the efficacy of read-through agents to induce CFTR function in organoids with a PTC. Next to swelling, we analyzed the luminal expansion upon forskolin stimulation as percentage of total organoid area, which is found to be more sensitive than FIS (Fig. 1, 2). Forskolin-induced luminal expansion precedes organoid swelling, which allows to analyze compounds with limited yet differential efficacy. The dynamic range, however, is limited and quickly reaches a ceiling; when organoids expand to a greater extent, the ratio of luminal area and total organoid area will not change that much.

G418 induced FIS and luminal expansion in a stop codon mutation-specific manner, demonstrating that read-through by pharmacological agents can be detected in organoids (Fig. 1, 2). The G418-induced CFTR function was further stimulated by CFTR-modifying drugs VX-770 and VX-809 in organoids lacking residual CFTR function (*E60X/4015delATTT*), confirming G418 induced full-length CFTR protein and confirming previous data [22] (Fig. 1A, C-D). The efficacy of G418+VX-770+VX-809 was within the range associated with residual CFTR function of *F508del* homozygous organoids (Fig. 1C). These data indicated that *E60X/4015delATTT* intestinal organoids express sufficient *CFTR* nonsense mRNA levels for detection of read-through by pharmacological agents such as G418. The effect of G418-induced read-through on CFTR function in nonsense/F508del organoids was significant, yet very small compared to CFTR-targeting drugs (Fig. 2).

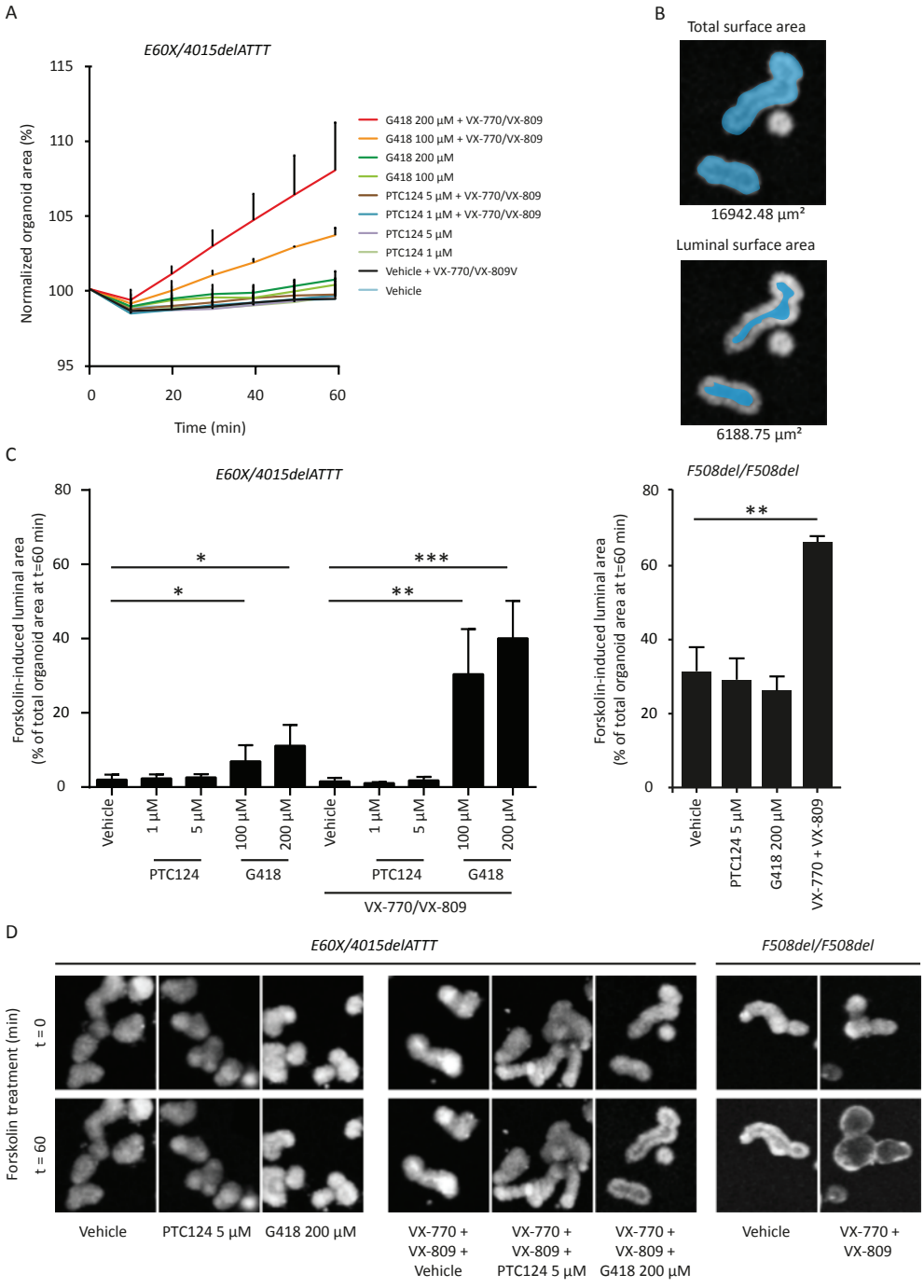

Figure 1: Correction of CFTR-PTC in intestinal organoids by G418 but not PTC124
Forskolin-induced swelling (FIS) of intestinal organoids (E60X/4015delATTT and F508del/F508del), incubated with either G418, PTC124 or DMSO (vehicle), w/o VX-809 for 24h and stimulated with forskolin, w/o VX-770. Conditions

were measured in triplicate at three independent time points for each patient. Concentrations were used as indicated. (a) Normalized E60X/4015delATTT organoid area increase in 60 min of FIS (%, ±SD); (b) representative images to determine total area and luminal area per well; (c) luminal size (% of total organoid area) after 60 minutes of FIS (%, ±SD), *p<0.05, **p<0.01; D) representative confocal images, conditions as indicated.

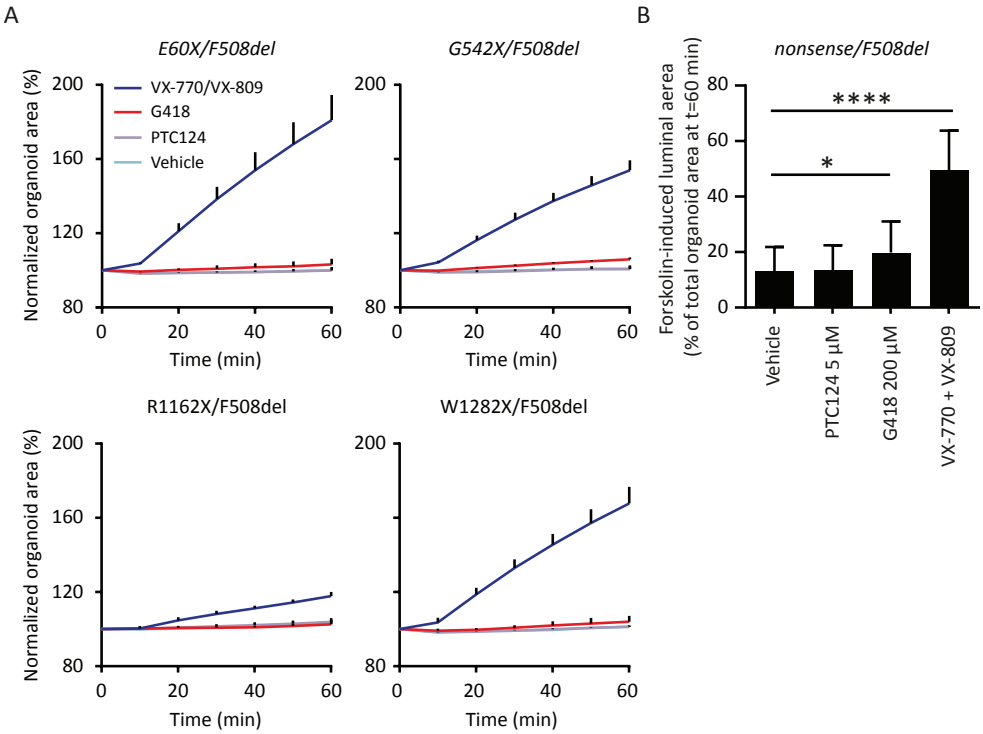

Figure 2: Marginal effect of G418 and PTC124 compared to CFTR-targeting drugs
Forskolin-induced swelling of nonsense/F508del intestinal organoids (E60X, G542X, R1162X and W1282X), incubated with either G418 (200 µM), PTC124 (5 µM) or DMSO (vehicle), w/o VX-809 (3 µM) for 24h. Forskolin-induced swelling (5 µM, w/o VX-770 (1 µM)) was monitored for 60 minutes. Conditions were measured in triplicate and in three independent experiments for each patient. (a) Normalized organoid area of PTC/F508del organoids (%, ±SD); (b) Forskolin-induced luminal area as percentage of total organoid area at t=60 min (±SD), pooled for all four genotypes of Fig. 2A, *p<0.05, ***p<0.001.

In contrast, PTC124 did not induce read-through in combination with VX-770 and VX-809 (Fig. 1A, C-D). Surprisingly, no read-through was observed when organoids were stimulated with PTC124 alone (Fig. 1A, 2A). We tested two compound batches of PTC124, and included *CFTR* mutations that were previously associated with PTC124 read-through, such as G542X (c.1624G->T) and W1282X (c.3846G->A) (cftr2.org). Moreover, removal of antibiotics from the culture medium and inhibition of nonsense RNA-mediated decay by amlexanox pretreatment [10] did not increase G418-induced or PTC124-induced read-through (data not shown). We did not control for uptake of the read-through agents by organoids that might be limited due to specific culture conditions or selective activity of drug pumps. Despite our inability to detect PTC124 activity, it remains possible that some individuals may be identified who show response to PTC124. Also, the efficacy of PTC124 might be tissue- and/or species-specific, although PTC suppression by PTC124 was observed

in mouse-intestine [9].

The inability to detect read-through by PTC124 in organoids is consistent with recently published in vitro data [13] and the lack of efficacy in phase 3 clinical trials in both CF [16] and DMD [14,19]. However, data are conflicting with reports indicating subject-specific PTC124 activity in open-label phase 2 clinical trials as suggested from CFTR biomarker analysis [15,17-18], and the retrospective analysis of the phase 3 clinical trial data suggesting mild clinical efficacy of PTC124 in tobramycin-free subjects [16]. Our data would support the development of more effective read-through compounds in combination with CFTR-protein restoring drugs

Conflict of interest

JFD, CKE and JMB are inventors on a patent application related to these findings.

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Acknowledgements

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Reference List

1 Riordan, J. R. CFTR function and prospects for therapy. *Annu Rev Biochem* **77**, 701-726, (2008).
2 Sosnay, P. R. *et al.* Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene. *Nat Genet* **45**, 1160-1167, (2013).
3 Van Goor, F. *et al.* Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A* **106**, 18825-18830, (2009).
4 Van Goor, F. *et al.* Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proc Natl Acad Sci U S A* **108**, 18843-18848, (2011).
5 De Boeck, K., Zolin, A., Cuppens, H., Olesen, H. V. & Viviani, L. The relative frequency of CFTR mutation classes in European patients with cystic fibrosis. *J Cyst Fibros* **13**, 403-409, (2014).
6 Howard, M., Frizzell, R. A. & Bedwell, D. M. Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. *Nat Med* **2**, 467-469, (1996).
7 Keeling, K. M., Wang, D., Conard, S. E. & Bedwell, D. M. Suppression of premature termination codons as a therapeutic approach. *Crit Rev Biochem Mol Biol* **47**, 444-463, (2012).
8 Welch, E. M. *et al.* PTC124 targets genetic disorders caused by nonsense mutations. *Nature* **447**, 87-91, (2007).
9 Du, M. *et al.* PTC124 is an orally bioavailable compound that promotes suppression of the human CFTR-G542X nonsense allele in a CF mouse model. *Proc Natl Acad Sci U S A* **105**, 2064-2069, (2008).
10 Gonzalez-Hilarion, S. *et al.* Rescue of nonsense mutations by amlexanox in human cells. *Orphanet J Rare Dis* **7**, 58, (2012).
11 Auld, D. S. *et al.* Molecular basis for the high-affinity binding and stabilization of firefly luciferase by PTC124. *Proc Natl Acad Sci U S A* **107**, 4878-4883, (2010).
12 Auld, D. S., Thorne, N., Maguire, W. F. & Inglese, J. Mechanism of PTC124 activity in cell-based luciferase assays of nonsense codon suppression. *Proc Natl Acad Sci U S A* **106**, 3585-3590, (2009).
13 McElroy, S. P. *et al.* A lack of premature termination codon read-through efficacy of PTC124 (Ataluren) in a diverse array of reporter assays. *PLoS Biol* **11**, e1001593, (2013).
14 Finkel, R. S. *et al.* Phase 2a study of ataluren-mediated dystrophin production in patients with nonsense mutation Duchenne muscular dystrophy. *PLoS One* **8**, e81302, (2013).

15 Kerem, E. *et al.* Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial. *Lancet* **372**, 719-727, (2008).
16 Kerem, E. *et al.* Ataluren for the treatment of nonsense-mutation cystic fibrosis: a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Respir Med* **2**, 539-547, (2014).
17 Sermet-Gaudelus, I. *et al.* Ataluren (PTC124) induces cystic fibrosis transmembrane conductance regulator protein expression and activity in children with nonsense mutation cystic fibrosis. *Am J Respir Crit Care Med* **182**, 1262-1272, (2010).
18 Wilschanski, M. *et al.* Chronic ataluren (PTC124) treatment of nonsense mutation cystic fibrosis. *Eur Respir J* **38**, 59-69, (2011).
19 Hoffman, E. P. & Connor, E. M. Orphan drug development in muscular dystrophy: update on two large clinical trials of dystrophin rescue therapies. *Discov Med* **16**, 233-239, (2013).
20 Dekkers, J. F. *et al.* A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* **19**, 939-945, (2013).
21 Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-265, (2009).
22 Xue, X. *et al.* Synthetic aminoglycosides efficiently suppress cystic fibrosis transmembrane conductance regulator nonsense mutations and are enhanced by ivacaftor. *Am J Respir Cell Mol Biol* **50**, 805-816, (2014).

4

A bioassay using intestinal organoids to measure CFTR modulators in human plasma

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Abstract

Treatment efficacies of drugs depend on patient-specific pharmacokinetic and pharmacodynamic properties. Here, we developed an assay to measure functional levels of the CFTR potentiator VX-770 in human plasma and observed that VX-770 in plasma from different donors induced variable CFTR function in intestinal organoids. This assay can help to understand variability in treatment response to CFTR potentiators by functionally modeling individual pharmacokinetics.

Introduction

The CFTR potentiator VX-770 (ivacaftor) is registered for the CFTR gating mutation CFTR-G551D and recently also for eight other gating mutations including CFTR-S1251N [1-3]. VX-770 treatment shows exciting and robust treatment effects at the group level, but treatment effects between individuals are variable [4-7]. This variability remains difficult to predict and mechanisms are poorly understood, especially in subjects with identical CF causing mutations. Differences in pharmacokinetics likely play a role in modifying the individual efficacy of VX-770. Plasma C_{max} of about 3.5 µM are reached after 5 days of VX-770 150 mg twice a day (NDA 203188; www.fda.gov). Most VX-770 is bound to albumin and alpha 1-acid glycoprotein in blood, known factors modifying this interaction in blood include fatty diets, inflammation and infection [8]. VX-770 is metabolized into functional and non-functional metabolites primarily in a CYP3A4-dependent fashion, and excreted mainly via feces (EMEA/H/C/002494//0000)[9]. To better characterize the pharmacokinetic details of VX-770 at the level of an individual patient, we developed an assay to functionally measure VX-770 in plasma of healthy volunteers using a recently developed functional CFTR assay in organoids [10-14].

Materials and Methods

Collection of human materials - Human plasma and rectal organoids were obtained after written informed consent, according to the principles of the declaration of Helsinki.

Human plasma preparation - Healthy control peripheral blood was collected using sodium-heparin Vacuette tubes (Greiner Bio-one) and cooled for 10 min on ice. Plasma was isolated after centrifugation (560 g for 10 min at 4 °C) and stored at -80 °C until use. Plasma was thawed and VX-770 or VX-809 (Selleck Chemicals) was introduced at various concentrations.

CFTR function measurements in organoids - Intestinal crypts were isolated from cystic fibrosis patients (R117H/F508del, S1251N/F508del and F508del/F508del), and CFTR function was quantified by measurement of forskolin-induced swelling of intestinal organoid cultures as previously described [10]. Organoids were cultured for at least 3 weeks after isolation. Organoids were stimulated directly with VX-770 or for 20 h with VX-809, diluted in plasma as indicated in the text.

Results

CFTR-S1251N potentiation by VX-770 in pooled plasma

To determine assay conditions that allow us to discriminate between relevant concentrations of VX-770 in plasma, we stimulated organoids expressing the gating mutant CFTR-S1251N with various amounts of pooled plasma from ten healthy individuals in combination with VX-770 concentrations. We first analyzed various anti-coagulants and found heparin-based plasma most optimal for FIS assays (citrate and EDTA affected the Matrigel structure, serum was also suited for FIS assays, Fig. S1A and B). We stimulated organoids with a suboptimal amount of forskolin (0.4 µM) to facilitate optimal detection of VX-770-induced CFTR function since considerable residual function associated with S1251N at high forskolin levels limits the VX-770-dependent swelling (Fig S2A, B). We observed a VX-770 dose-dependent increase of forskolin-induced organoid swelling, and no effects of plasma on swelling of organoids when added up to 10% of the total assay volume (Fig. 1A, B). The extrapolation of these plasma and VX-770 concentrations to 100% plasma indicated

that VX-770 could be measured between 0.25 and 40 µM in 100% plasma, exceeding ranges associated with *in vivo* treatment (NDA 203188; www.fda.gov). We observed that VX-770 concentrations associated with the normal treatment range between patients could be clearly differentiated when organoids were stimulated with serially diluted plasma (0.75-6%) containing introduced VX-770 at 0.5, 1.5 or 4 µM in 100% plasma (Fig. 1C).

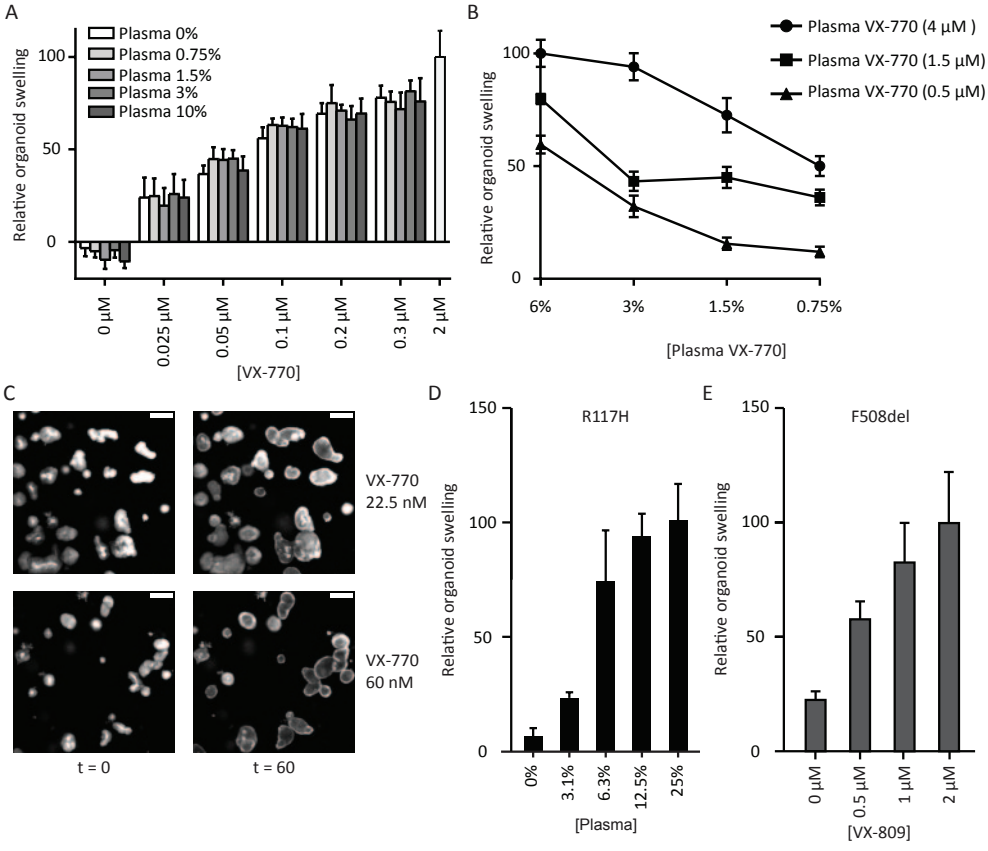


Figure 1. Functional activity of CFTR modulators in plasma.
(a) Pooled plasma from ten healthy donors, VX-770 and forskolin (0.4 µM) were combined to stimulate CFTR-S1251N expressing organoids. Swelling relative to t = 0 min was measured for 60 min, and area under the curve (AUC) data were calculated and normalized to stimulation with 2 µM of VX-770 in medium. Data were measured at three independent time points in triplicate and presented as mean (±SEM). (b) Normalized AUC measurements of organoid swelling after stimulation with serial dilutions of pooled plasmas containing VX-770 (0.5, 1.5 and 4 µM in 100% plasma) using 0.4 µM forskolin. Data were measured at three independent time points in triplicate and presented as mean (±SEM). (c) Representative examples of organoids from (b) at t = 0 and t = 60 min for indicated conditions (scale bar indicates 250 µm). (d) Swelling of R117H organoids after stimulation with forskolin (0.05 µM) and serial dilution of plasma containing VX-770 (2 µM in 100% plasma). Single experiment, measured in triplicate data presented as mean (±SD). (e) Forskolin-induced (5 µM) swelling of F508del organoids after 20h incubation with 10% plasma and VX-809 (0, 0.5, 1 or 2 µM). Data were measured in triplicate in four independent experiments and presented as mean (±SEM).

Next, we investigated whether other CFTR-mutations also responds to CFTR modulators suspended in plasma. Stimulating CFTR-R117H organoids with forskolin (0.05 μM) and increasing amounts of plasma containing VX-770 (2 μM in 100% plasma) resulted in a dose dependent increase in organoid swelling (Fig. 1D). Furthermore, overnight incubation of CFTR-F508del organoids with 10% plasma and increasing VX-809 concentrations (0.5, 1 and 2 μM) dose dependently increased forskolin (5 μM)-induced CFTR function (Fig. 1E). Collectively, these data indicate the FIS-assay can discriminate between clinically-associated concentrations of VX-770 in plasma and can also be used to detect other CFTR modulating drug in plasma.

Variability of VX-770-dependent swelling between plasma samples

We investigated the variability of VX-770-dependent organoid swelling in plasma samples derived from 9 different healthy donors in the presence of VX-770 on CFTR-S1251N expressing organoids. Forskolin induced swelling of organoids was assessed after addition of 6% plasma with or without 60 nM VX-770 representing 1 μM in 100% plasma. The ability of these plasmas to stimulate organoid swelling was different between samples (Fig. 2). Without addition of VX-770, 6% of plasma induced no swelling as observed earlier, albeit that limited organoid swelling was consistently observed using plasma of donor 8. The introduction of a similar amount of VX-770 to these plasma samples resulted in different swell rates between samples, with the majority of responses not different from the average but reduced VX-770 dependent swelling was observed for donor 1 and enhanced VX-770-dependent swelling was observed for donor 8. These data indicated that VX-770-dependent organoid swelling is modified by individual plasma.

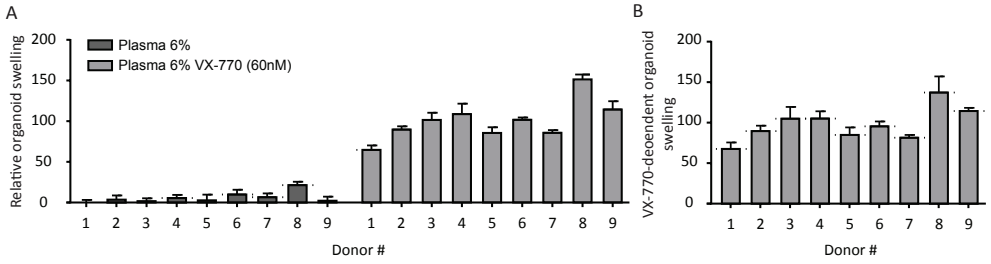


Figure 2. Variation in VX-770 dependent organoid swelling between plasma samples.
(a) CFTR-S1251N expressing organoids were stimulated with 6% plasma from 9 different donors in the absence or presence of VX-770 (60 nM, representing 1 μM in 100% plasma) and forskolin (0.5 μM). Individual data points were normalized to the average VX-770 response of the 9 plasma samples. (b)The VX-770-dependent part of organoid swelling was calculated by subtraction of the plasma response from the plasma + VX-770 response per plasma in (a). Data were normalized to the average of all data points. All data were measured at three independent time points in triplicate and presented as mean (±SEM).

Discussion

In this report we describe a bioassay to measure CFTR modulator activity in human plasma using intestinal organoids. The bioassay described here was found suited to discriminate between differences in VX-770 concentrations that were observed after treatment in patients with CFTR-gating mutations and can also be used to study CFTR correctors. Importantly, we also observed that the VX-770-dependent organoid swelling was different between plasma samples of healthy controls after introducing identical amounts of

VX-770 and stimulation of identical organoids. This could indicate that plasma components may modulate the functional activity of VX-770 and that the absolute VX-770 concentration in plasma does not necessarily determine its functional activity. The exact mechanisms for these observed interactions between plasma components, VX-770 and the intestinal organoids require further exploration.

This plasma-based assay is a useful tool to understand mechanisms controlling the functional level of CFTR modulators in blood. CFTR potentiator treatment *in vivo* is variable and likely dependent of patient-specific variability in absorption, distribution, metabolism and excretion [4-6]. Although the function of potentiators in plasma will possibly not fully represent the functional activity of these drugs in the tissue, peripheral blood is easily accessible and, as shown here, would allow the functional modeling of an individual's pharmacokinetic profile after potentiator treatment. Relations between plasma-induced CFTR function before and during treatment, and clinical observations in various CFTR-expressing tissues are required to validate the use of this assay to better predict drug efficacy at the level of the individual. Such an approach may further be personalized when the patient's plasma is used to stimulate the patient's own intestinal organoids as a combined model to functionally integrate individual parameters controlling pharmacokinetics and pharmacodynamics.

Conflict of interest

CKE and JMB are inventors on a patent application related to these findings.

Acknowledgements

This work was part of the HIT-CF program, supported by grants of the Dutch Cystic Fibrosis Foundation (NCFS) and the Wilhelmina Children's Hospital (WKZ) Foundation, The Netherlands. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Reference List

1 Vertex Pharmaceuticals Incorporated. *U.S. Food and Drug Administration Approves KALYDECO® (ivacaftor) for Use in People with Cystic Fibrosis Ages 6 and Older Who Have the R117H Mutation*, 2014).

2 Yu, H. *et al.* Ivacaftor potentiation of multiple CFTR channels with gating mutations. *J Cyst Fibros* **11**, 237-245, (2012).

3 Van Goor, F. *et al.* Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A* **106**, 18825-18830, (2009).

4 Accurso, F. J. *et al.* Effect of VX-770 in persons with cystic fibrosis and the G551D-CFTR mutation. *N Engl J Med* **363**, 1991-2003, (2010).

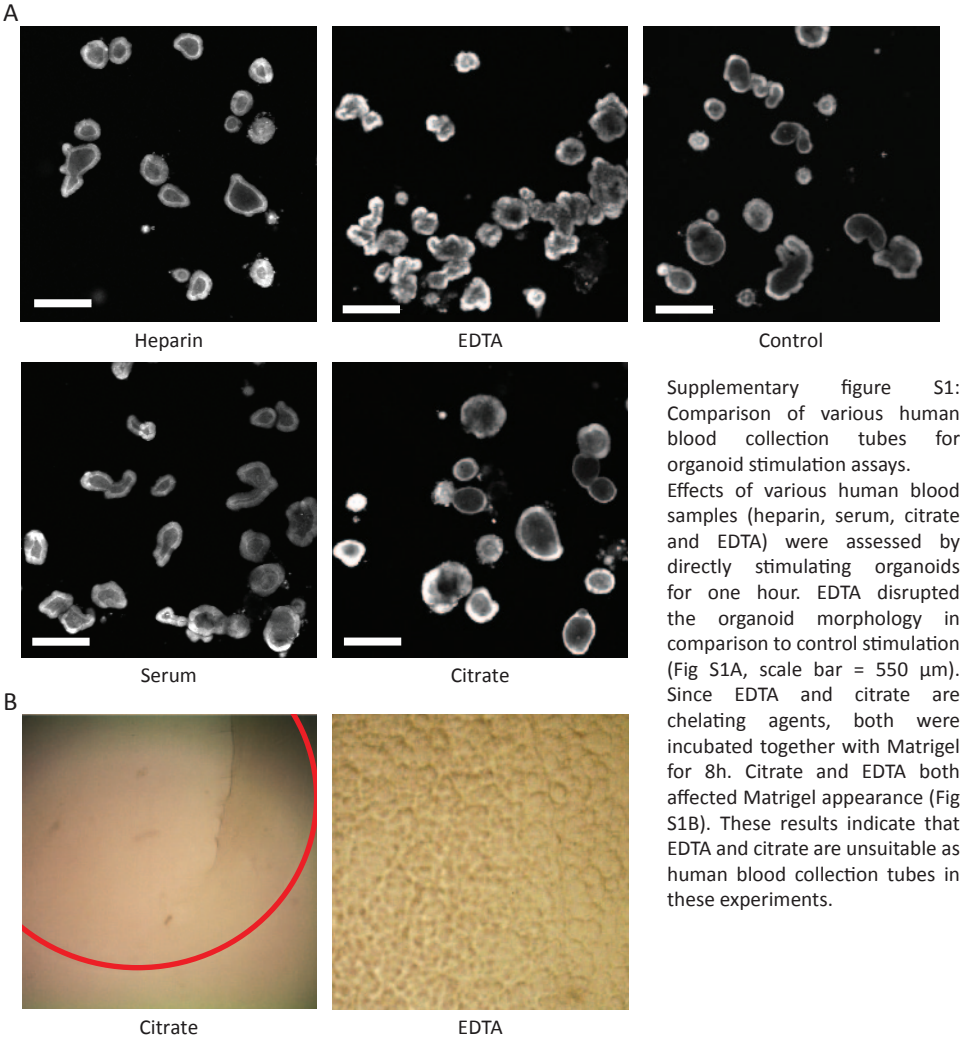
5 Ramsey, B. W. *et al.* A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *N Engl J Med* **365**, 1663-1672, (2011).

6 Rowe, S. M. *et al.* Clinical mechanism of the cystic fibrosis transmembrane conductance regulator potentiator ivacaftor in G551D-mediated cystic fibrosis. *Am J Respir Crit Care Med* **190**, 175-184, (2014).

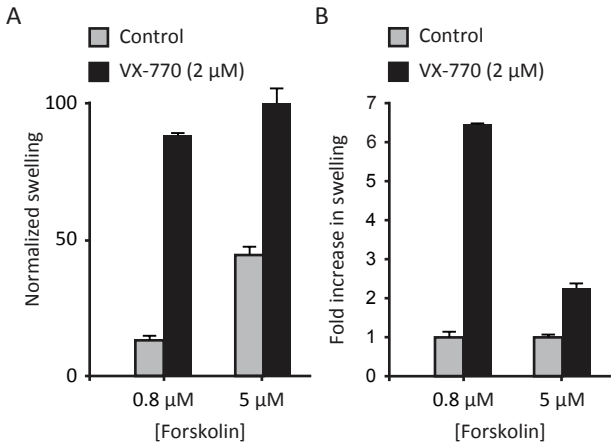
7 Flume, P. A. *et al.* Ivacaftor in subjects with cystic fibrosis who are homozygous for the F508del-CFTR mutation. *Chest* **142**, 718-724, (2012).

8 Israili, Z. H. & Dayton, P. G. Human alpha-1-glycoprotein and its interactions with drugs. *Drug Metab Rev* **33**, 161-235, (2001).

9 Mouly, S. J., Paine, M. F. & Watkins, P. B. Contributions of CYP3A4, P-glycoprotein, and serum protein binding to the intestinal first-pass extraction of saquinavir. *J Pharmacol Exp Ther* **308**, 941-948, (2004).
10 Dekkers, J. F. *et al.* A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* **19**, 939-945, (2013).
11 Dekkers, J. F., van der Ent, C. K. & Beekman, J. M. Novel opportunities for CFTR-targeting drug development using organoids. *Rare Dis* **1**, e27112, (2013).
12 Jung, P. *et al.* Isolation and in vitro expansion of human colonic stem cells. *Nat Med* **17**, 1225-1227, (2011).
13 Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762-1772, (2011).
14 Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-265, (2009).



Supplementary figure S1: Comparison of various human blood collection tubes for organoid stimulation assays.
Effects of various human blood samples (heparin, serum, citrate and EDTA) were assessed by directly stimulating organoids for one hour. EDTA disrupted the organoid morphology in comparison to control stimulation (Fig S1A, scale bar = 550 µm). Since EDTA and citrate are chelating agents, both were incubated together with Matrigel for 8h. Citrate and EDTA both affected Matrigel appearance (Fig S1B). These results indicate that EDTA and citrate are unsuitable as human blood collection tubes in these experiments.



Supplementary figure S2: Forskolin and VX-770 induced swelling on S1251N organoids.
At low forskolin (0.8 µM) concentration the induced S1251N organoid swelling was markedly less compared to high forskolin (5 µM) (Fig S2A). The maximal amount of swelling when combined with VX-770 (2 µM), was lower for lower forskolin concentrations. However, the fold increase in organoids swelling was far greater at lower forskolin concentrations (Fig S2B). Therefore, low forskolin concentration were used in the experiments described.

5

β2-Adrenergic receptor agonists activate CFTR in intestinal organoids and subjects with cystic fibrosis

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Abstract

We hypothesized that people with cystic fibrosis (CF) who express *CFTR* (cystic fibrosis transmembrane conductance regulator) gene mutations associated with residual function may benefit from G-protein coupled receptor (GPCR)-targeting drugs that can activate and enhance CFTR function. We used intestinal organoids to screen a GPCR-modulating compound library and identified β2-adrenergic receptor agonists as the most potent inducers of CFTR function. β2-Agonist-induced organoid swelling correlated with the CFTR genotype, and could be induced in homozygous CFTR-F508del organoids and highly differentiated primary CF airway epithelial cells after rescue of CFTR trafficking by small molecules. The *in vivo* response to treatment with an oral or inhaled β2-agonist (salbutamol) in CF patients with residual CFTR function was evaluated in a pilot study. 10 subjects with a R117H or A455E mutation were included and showed changes in the nasal potential difference measurement after treatment with oral salbutamol, including a significant improvement of the baseline potential difference of the nasal mucosa (+6.4 mV, $p<0.05$), suggesting that this treatment might be effective *in vivo*. Furthermore, plasma that was collected after oral salbutamol treatment induced CFTR activation when administered *ex vivo* to organoids. This proof-of-concept study suggests that organoids can be used to identify drugs that activate CFTR function *in vivo* and to select route of administration.

Introduction

The *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene encodes an apical anion channel and is mutated in subjects with cystic fibrosis (CF) [1]. Subjects with CF have an altered composition of many mucosal surface fluids, leading to dysfunction of the gastro-intestinal and pulmonary systems as well as other organs. The most common mutation is a deletion of phenylalanine at position 508 (p.Phe508del; F508del) and is present in approximately 90% of subjects with CF, of which ~65% is F508del homozygote (www.genet.sickkids.on.ca). CF disease expression is highly variable between subjects due to the complex relations between *CFTR* genotype, modifier genes and environmental factors, which are unique for each individual [2-6].

Approximately 2000 *CFTR* mutations have been described, which are divided into different classes according to their impact on CFTR expression and function [7]. Briefly, class I mutations result in no functional protein (e.g. stop codons and frame shifts), class II mutations severely affect apical trafficking (e.g. F508del), class III mutations disrupt channel regulation or gating (*e.g.* p.Gly551Asp; G551D), class IV mutations reduce channel conductance (*e.g.* p.Arg334Trp; R334W), class V mutations lead to reduced apical expression of normally-functioning CFTR (e.g. p.Ala455Glu; A455E), and class VI mutations accelerate CFTR turnover at the plasma membrane. Whereas class I-III and VI mutations are generally associated with no or very limited residual function, some residual function is associated with class IV and V mutations and milder disease phenotype such as CFTR-A455E and CFTR-R117H (p.Arg117His; shared class III and IV) (www.cftr2.org).

Novel drugs are being developed to target mutation-specific CFTR defects. The potentiator VX-770 (ivacaftor) enhances the activity of apical CFTR and was shown to provide clinical benefit for patients with CFTR gating mutations [8-10]. Pharmacological repair of CFTR-F508del has proven more difficult, although encouraging phase III clinical trial results have been reported for CFTR-F508del homozygous subjects treated with a combination of ivacaftor and the corrector lumacaftor (VX-809) [11], which partly restores trafficking of CFTR-F508del to the apical membrane [12]. However, the therapeutic effects of these therapies are variable between subjects, and remain insufficient to fully restore CF and CFTR-related disease markers, indicating that more effective treatments are still required.

Individual CFTR function depends on endogenous signaling pathways that control its channel function. Various endogenous ligands have been identified which activate CFTR in a cAMP/protein kinase A-dependent fashion. Many of these ligands (*e.g.* vasoactive intestinal peptide, prostaglandins and β-adrenergic stimuli) signal by binding to G-protein coupled receptors (GPCRs), which releases cytosolic G-proteins that activate adenylyl cyclase to generate cAMP [13-15]. While it is known that tissue-specific activity of CFTR is regulated by diverse ligands, the extent to which CFTR function is limited by cAMP production is not clear.

We hypothesized that cAMP-dependent signaling is a rate-limiting step for CFTR activation *in vivo* and that CF individuals who express alleles associated with residual function might benefit from existing drugs that stimulate cAMP. Therefore, we screened a small chemical compound library of GPCR modulators for their ability to stimulate (mutant) CFTR activity in primary rectal organoids from healthy control and CF subjects. Rectal organoids grow from intestinal stem cells and self-organize into multi-cellular three-dimensional structures consisting of a single epithelial layer, with the apical membrane facing a closed

central lumen [16-18]. Addition of forskolin, which raises cAMP, stimulates CFTR-dependent fluid secretion into the organoid lumen and induces rapid organoid swelling [19-20]. We here provide proof-of-concept that intestinal organoids can be used as tool to identify potential drugs and route of administration for particular CF subgroups.

Materials and methods

Human participants - This study was approved by the Ethics Committee of the University Medical Centre Utrecht and the Erasmus Medical Centre Rotterdam. Informed consent was obtained from all subjects. Organoids from healthy controls and cystic fibrosis subjects were generated from rectal biopsies after intestinal current measurements obtained (i) during standard CF care, (ii) for diagnostic purposes or (iii) during voluntary participation in studies.

Materials - The GPCR compound library, VX-809 and VX-770 were purchased from SelleckChem (Houston, TX, USA). Carvedilol, forskolin, salbutamol, salmeterol, terbutaline, epinephrine, ritodrine, dimethyl sulfoxide (DMSO), *N*-acetylcysteine, nicotinamide and SB202190 were purchased from Sigma (St. Louis, MO, USA). Formoterol was purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). $CFTR_{inh}$-172 was obtained from the CFF Therapeutics (Chicago, IL, USA). Matrigel was purchased from BD (Franklin lakes, NJ, USA). Calcein AM, supplements N-2 and B-27, Glutamax, advanced Dulbecco's modified Eagle medium/Ham's F-12 (DMEM/F-12), penicillin/streptomycin, HEPES and murine epidermal growth factor (mEGF) were purchased from Life Technologies (Bleiswijk, The Netherlands). A83-01 was purchased from Tocris (Abingdon, UK). TOPflash and FOPflash were purchased from Millipore (Amsterdam, The Netherlands).

Human organoid cultures - Rectal crypt isolation and organoid expansion was performed with some adaptations of previously described methods [20-21]. Briefly, rectal biopsies were thoroughly washed with PBS and incubated in 10 mM EDTA for 90 minutes at 4°C. The crypts were collected by centrifugation and suspended in 50% Matrigel and 50% complete culture medium (advanced DMEM/F-12 media supplemented with penicillin/streptomycin, HEPES, GlutaMax, supplements N-2 and B-27, *N*-acetylcysteine, nicotinamide, mEGF, A83-01, SB202190, 50% Wnt3a, 20% Rspo-1 and 10% Noggin conditioned media) that was allowed to solidify at 37°C for 20 minutes in three droplets of 10 µL per well of a 24-wells plate. The droplets were then immersed in pre-warmed complete culture medium and cultures were expanded for at least 3 weeks before assaying CFTR function. Complete culture medium was refreshed three times per week and organoids were passaged weekly. Quality of the conditioned media was assessed by dot blots, ELISA and luciferase reporter constructs (TOPflash and FOPflash) [22-23].

GPCR compound library - The GPCR small molecule compound library comprises 61 agonists and antagonists that target a wide range of GPCR families including adrenergic, dopamine, opioid, serotonin, histamine and acetylcholine receptors. A complete list of chemicals in the library is given in Supplementary Table S1.

CFTR function measurement in organoids - Organoids were reseeded 1 day before functional analysis in 96-well plates as described previously [20]. CFTR-F508del organoids were incubated with VX-809 (3 µM) for 24 h, as indicated in text and figure legends. Organoids were stained with Calcein green AM (2.5 µM) 1 h prior the addition of compound, and each compound was tested at four different concentrations (10, 2, 0.4 and 0.08 µM). Forskolin (5 µM) and DMSO were used as positive and negative controls, respectively. Organoid swelling was monitored for 1 h using a ZEISS LSM 710 confocal microscope (Zeiss, Jena, Germany). The relative increase in surface area was calculated using Volocity (version 6.1.1; PerkinElmer, Waltham, MA, USA). The area under the curve was calculated as described previously [20]. Carvedilol (10 µM) was incubated for 30 min prior to stimulation and organoids were pretreated with $CFTR_{inh}$-172 (150 µM) for 4 h to inhibit CFTR-dependent responses.

Halide sensitive YFP quenching in CFBE41o- cells - CFBE41o$^-$ cell lines overexpressing CFTR-F508del or CFTR-WT were grown in α- minimal essential medium containing 8% heat-inactivated fetal calf serum, penicillin and streptomycin at 37°C in a humidified 5% CO_2 incubator as described [24-25]. CFBE41o- cells were transduced with the ratiometric halide-sensitive pHAGFE2-YFP (46L-148Q-152L)-mKATE sensor for measurement of CFTR activity as described previously [26]. Briefly, cells were incubated for 24 h with VX-809 (10 µM). After 20 min stimulation in a chloride-containing buffer, the cells were washed with iodide buffer and the decrease in fluorescence was monitored using a ZEISS LSM 710 microscope for 60 s. The rate of YFP (yellow fluorescent protein)/mKate quenching was calculated using Prism 6 (GraphPad Software, La Jolla, CA, USA).

Ussing chamber measurements in primary airway epithelial cells - Primary F508del/F508del human bronchial epithelial cells from the Primary Airway Cell Biobank of the CF Translational Research centre at McGill University were cultured at the air/liquid interface for 3 weeks and pre-treated for 24 h with VX-809 (1 μM). Control monolayers from the same patients were handled similarly but exposed to vehicle (0.1% DMSO) during the pre-treatment period. For electrophysiological measurements, monolayers were mounted in Ussing chambers (EasyMount; Physiologic Instruments, San Diego, CA, USA) and voltage-clamped using a VCCMC6 multichannel current-voltage clamp (Physiologic Instruments, San Diego, CA, USA). The voltage clamp was connected to a PowerLab/8SP interface for data collection (ADInstruments, Colorado Springs, CO, USA) and analysis was performed using a PC as described previously [27]. Solutions were continuously gassed and stirred with 95% O_2/5% CO_2 and were maintained at 37°C by circulating water bath. Ag/AgCl reference electrodes were used to measure transepithelial voltage and pass current. Pulses (1 mV amplitude, 1 s duration) were delivered every 90 s to monitor resistance. A basolateral-to-apical Cl^- gradient was imposed and amiloride (100 μM) was added on the apical side to inhibit the epithelial sodium channel (ENaC) current. Monolayers were exposed acutely to 10 μM forskolin or salbutamol or to the vehicle 0.1% DMSO. After the short circuit current (I_{sc}) reached a plateau, a potentiator (either 50 μM genistein or 100 nM VX-770 as indicated) was added, followed by $CFTR_{inh}$-172 to confirm that the current responses were dependent on CFTR. Salbutamol was also assayed after pre-treatment with the antagonist carvedilol (10 μM) for 30 min as a further test of receptor specificity.

Pilot study with inhaled and oral salbutamol - In this open-label phase II pilot study, 10 patients were randomly assigned to receive four times daily 200 μg salbutamol per inhalation or four times daily 4 mg salbutamol orally, for 3 consecutive days (NTR4513). After a wash-out period of at least 4 days, patients received the opposite treatment. We included patients aged ≥18-years-old with a CFTR-A455E or a CFTR-R117H mutation on at least one allele of whom rectal biopsies and organoid cultures showed residual CFTR function in previous studies [20]. Patients were excluded if they had an acute pulmonary exacerbation or an increased risk of side-effects of salbutamol. The primary outcome measures were changes in sweat chloride concentration (SCC) and changes in Nasal Potential Difference (NPD) measurement, which are both *in vivo* biomarkers for CFTR function. The NPD and SCC measurements were performed according to the most recent version of the standard operating procedure of the European Cystic Fibrosis Society-Clinical Trials Network (www.ecfs.eu/ctn). The results of these measurements before and after treatments with salbutamol were compared using a Wilcoxon signed-rank test. A secondary outcome measure was the CFTR-activating capacity of the patients' plasma in organoids. Therefore, whole blood was collected in sodium-heparin tubes before treatment and after the last dose of salbutamol, when the maximum concentration of salbutamol in the blood was expected (inhaled salbutamol after 30 minutes, oral salbutamol after 2 hours; www.fk.cvz.nl). Plasma was isolated as described previously [28].

Patient plasma-induced organoid swelling - Patient plasma was collected before and after treatment with salbutamol and incubated (20% and 40% plasma) with organoids derived from a subject with CF with high residual function (R117H/F508del). Organoid swelling was monitored as described above. Reference values were generated by measurement of spiked salbutamol in 0%, 20% and 40% plasma.

Results

Screen for GPCR modulators of organoid swelling

To identify compounds that can activate CFTR, we assessed CFTR-dependent swelling of organoids in response to 61 GPCR-modulating compounds (Fig. 1) [20]. As observed previously, forskolin induced rapid swelling of CFTR-WT organoids, and to a lesser extent, of VX-809-treated homozygous CFTR-F508del organoids. As expected, DMSO did not induce swelling (Fig. 1a). Swelling was expressed as the area under the curve for each specific condition (Fig. 1a,b). Of the 61 compounds tested, dopamine, epinephrine, ritodrine and salbutamol dose-dependently induced swelling of CFTR-WT organoids, with highest potency for ritodrine and salbutamol and lowest potency for dopamine (Fig. 1c,d). Epinephrine, ritodrine and salbutamol are ligands for β_2-adrenergic receptors and dopamine for the dopamine receptor. At the highest dose, the response to the four compounds was comparable to the forskolin-induced swelling (Fig. 1d). In VX-809-corrected F508del

homozygous organoids, swelling was dose-dependently induced by epinephrine, salbutamol and ritodrine, but not by dopamine (Fig. 1c,e). The potency was highest for salbutamol, and lowest for ritodrine. High levels of salbutamol induced swelling to a similar extend as forskolin (Fig. 1d). In conclusion, β_2-adrenergic receptor stimulation can potently activate CFTR-WT and drug-corrected CFTR-F508del in organoids.

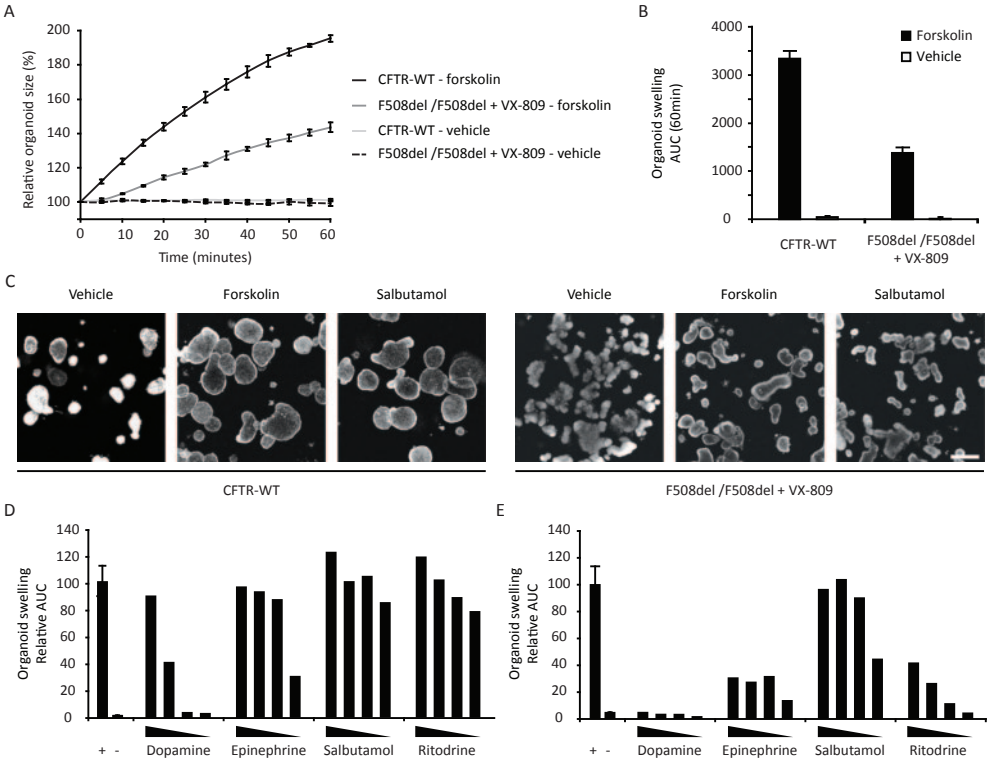

Figure 1. G-Protein coupled receptor (GPCR) modulator-induced swelling of healthy control and CFTR-F508del organoids.

(a) CFTR wild-type (WT) and F508del homozygous organoids were stimulated with forskolin (5 μM) or DMSO (vehicle, 0.05%) and the relative increase in size was monitored over 60 min. CFTR-F508del homozygous organoids were pre-incubated with VX-809 (3 μM) for 24 h. (b) Quantification of the area under the curve (AUC) of (a), baseline was set at 100%, t = 60 min. (c) Representative images of CFTR-WT and VX-809-corrected CFTR-F508del organoids after 60 minutes of stimulation with DMSO (vehicle), forskolin (5 μM) or salbutamol (10 μM). Scale bar is 200 μm. (d) Positive compounds for induction of fluid secretion using CFTR-WT intestinal organoids after screening a GPCR modulator library (61 compounds). Forskolin (+) and DMSO (-) were used as positive and negative control, respectively. GPCR modulators were tested at 10, 2, 0.4 and 0.08 μM. Data are normalized to the forskolin response. (e) Same compounds as in (d), tested on VX-809 (3 μM)-treated F508del homozygous organoids.

β_2-agonists robustly induce organoid swelling

Next, we assessed β_2-adrenergic receptor stimulation by short- and long-acting agonists in organoids with various *CFTR* mutations (Fig. 2). First, salbutamol- and ritodrine-induced swelling was confirmed in organoids derived from three individual F508del homozygous patients (Fig. 2a). As expected, robust organoid swelling was only observed after treatment with the CFTR-modulators VX-770 or VX-809, and was highest

upon VX-770 and VX-809 combination treatment. In line with figure 1, ritodrine was somewhat less potent than salbutamol and forskolin, especially for VX-809 incubated organoids. Both short-acting (ritodrine, terbutaline and salbutamol) and long-acting (formoterol, salmeterol and isoproterenol) β_2-agonists induced fluid secretion. Inhibition by CFTR-$_{inh}$172 or carvedilol supported CFTR or β-adrenergic receptor specificity, respectively (Fig. 2b). Forskolin and β_2-adrenergic receptor -induced swelling differed between organoids with distinct *CFTR* genotypes: we observed no swelling in organoids expressing two *CFTR-null* alleles (p.Glu60Ter and p.Ile1295fs; E60X and 4015delATTT), some swelling in CFTR-A455E or VX-809-corrected homozygous CFTR-F508del organoids, and high swelling in CFTR-R117H expressing organoids (Fig. 2c). Dose-dependencies of β_2-agonist-induced swelling were independent of the *CFTR* genotype or VX-809-rescued F508del, and indicated that long-acting β_2-agonists were most potent, whereas forskolin was least potent (Fig. 2d). Representative examples of agonist-induced swelling are indicated in figure 2e. Together, these data demonstrate that various β_2-agonists robustly induce CFTR function in a *CFTR* mutation-dependent manner.

Salbutamol-mediated CFTR activation in bronchial epithelial cells

To confirm that intestinal organoid responses can be relevant for airway epithelial cells, CFTR activation by the β_2-agonist salbutamol was studied in the CF airway cell line CFBE41o$^-$ (CFBE) and primary CF human bronchial epithelial (HBE) cells. First, we studied CFTR-dependent iodide quenching rates in CFBE cells that endogenously express CFTR-F508del and were previously transduced with *CFTR-F508del* (CFBE-F508del) or *CFTR-WT* (CFBE-CFTR-WT) cDNA [24]. To measure CFTR-dependent iodide-influx, the cells were stably transduced with a YFP/mKate sensor, as described previously [26]. Quenching of the YFP signal by iodide (indicating CFTR activity) was induced by both forskolin and salbutamol in VX-809+VX-770-treated CFTR-F508del and CFTR-WT CFBE cells (Fig. 3a). In addition, Ussing chamber experiments revealed that salbutamol and forskolin induced a CFTR-dependent short circuit current in F508del homozygous HBE cells treated with VX-809 and VX-770, but not in cultures without CFTR-repairing treatment (Fig. 3b-d). As expected, the response to forskolin and salbutamol was abolished by CFTR$_{inh}$-172 and the salbutamol-induced response was inhibited by carvedilol (Fig. 3c,d). To conclude, activation of modulator-repaired CFTR-F508del by β_2-agonists was recapitulated in respiratory cell lines and primary airway cultures.

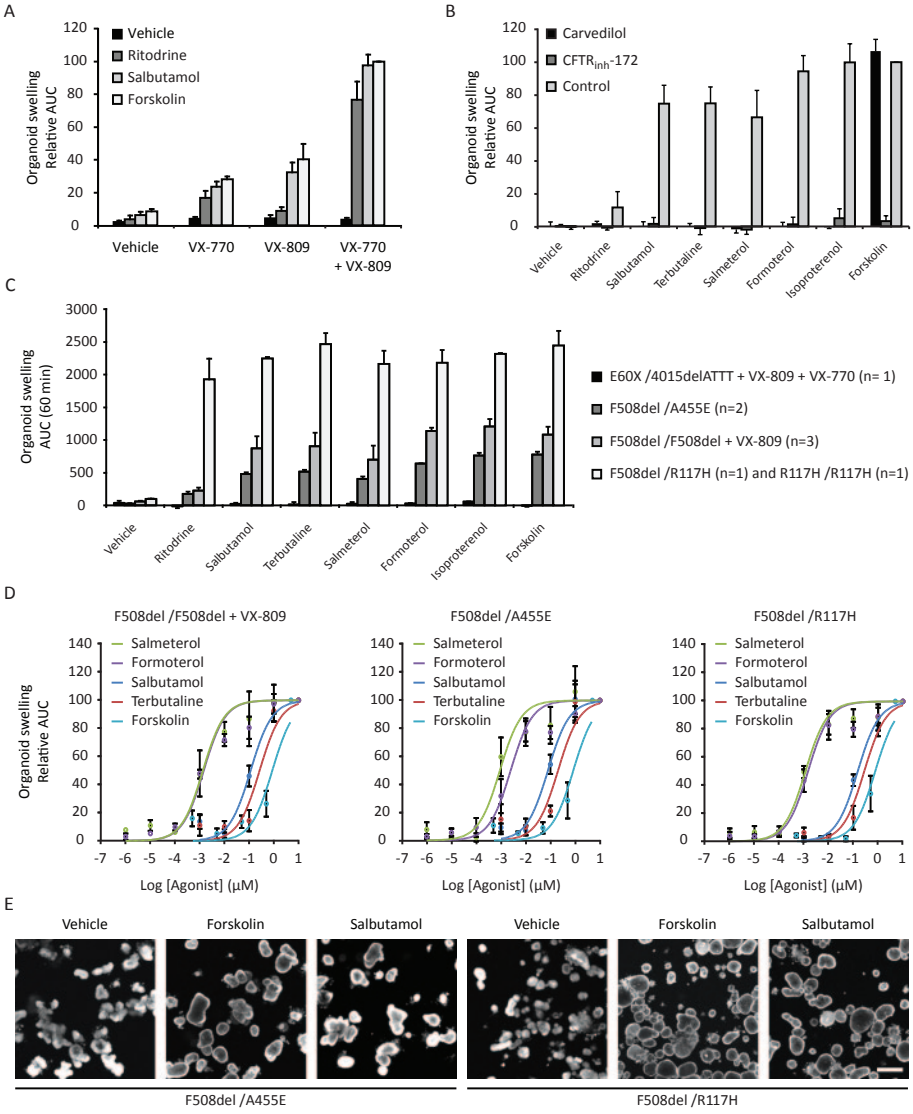

Figure 2. β_2-agonists induce CFTR activity.
(a) CFTR-F508del homozygous organoids were stimulated with ritodrine (10 μM), salbutamol (10 μM) or forskolin (5 μM). VX-809 (3 μM) was incubated for 24 h prior to stimulation. VX-770 (1 μM) was added simultaneously with the stimulus. Data were normalized to the combined VX-770+VX-809+ forskolin response, and organoids from three patients were measured at three independent time points in duplicate. Data are presented as mean±SEM. (b) VX-809 (3 μM) treated CFTR-F508del organoids were incubated with $CFTR_{inh}$-172 or carvedilol before stimulations. β_2-agonists were used at 10 μM and forskolin at 5 μM. All data were normalized to forskolin, and represent mean±SEM of three independent measurements in duplicate. (c) Organoids derived from patients with different *CFTR* genotypes were stimulated with β_2-agonists (10 μM) or forskolin (5 μM). n: number of patients, measured at three independent time points in duplicate (mean±SEM). (d) Dose-response curves for different β_2-agonists and forskolin in F508del/F508del, F508del/A455E and F508del/R117H organoids. All data were normalized to the highest concentration of stimulus, and represent mean±SEM of measurements at three independent time points. (e) Representative images of organoids expressing CFTR-F508del and either CFTR-A455E or CFTR-R117H after 60 minutes of stimulation with DMSO (vehicle), forskolin (5 μM) or salbutamol (10 μM). Scale bar: 200 μm.

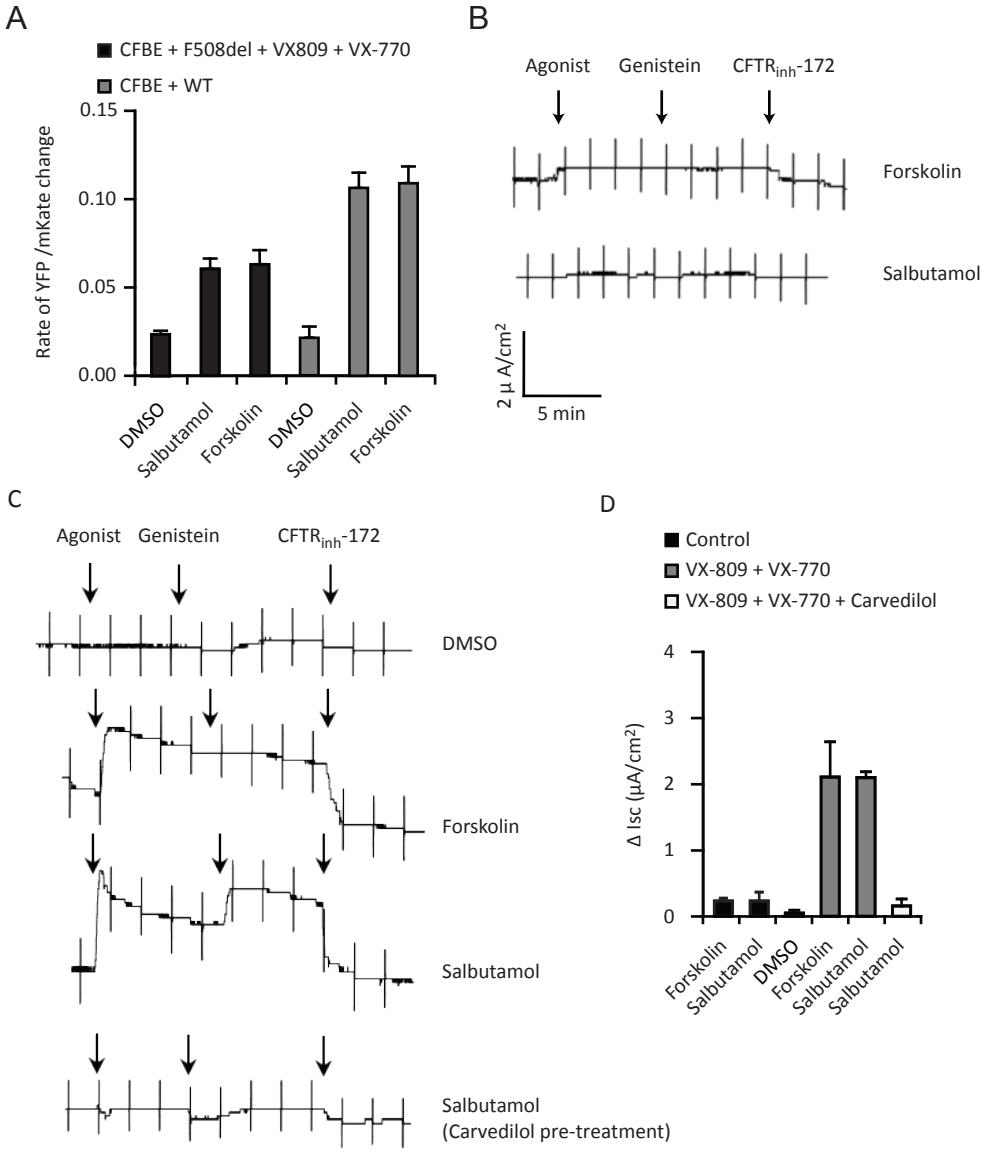

Figure 3. β_2-agonists-induced CFTR activation in bronchial epithelial cells.
(a) CFTR activity in CFBE41o$^-$ cells (CFBE) overexpressing CFTR-F508del or CFTR-WT, and stably expressing YFP/mKate, using an YFP quenching assay. CFBE41o$^-$ cells were pre-incubated for 24 h with VX-809 (10 µM), and stimulated with forskolin (25 µM) and VX-770 (10 µM) or salbutamol (10 µM) and VX-770 (10 µM) for 20 min prior to addition of iodide. Data are presented as mean±SEM and are representative of three independent experiments. (b) Highly differentiated primary CFTR-F508del bronchial epithelial cells cultured at the air/liquid interface were analyzed in Ussing chambers experiments. Representative traces of control-, forskolin- and salbutamol-stimulated conditions. Constant current pulses used to monitor transepithelial resistance cause the vertical deflections. (c) Representative Ussing chamber tracings for VX-809 (1 µM) and VX-770 (100 nM) treated CF bronchial epithelial cells, stimulated with DMSO, forskolin (10 µM) or salbutamol (10 µM). Scaling is identical to (b). (d) Quantification of (b) and (c), data are presented as mean±SEM and are representative of three independent experiments.

Pilot study with inhaled and oral salbutamol

To demonstrate that drugs identified by screens in rectal organoids can be used to modulate CFTR function *in vivo*, 10 CF patients were enrolled in a study, and treated with oral and inhaled salbutamol. One patient was only treated with oral salbutamol due to increased asthma symptoms during the wash-out period of salbutamol aerosol between both measurements. The baseline characteristics of the study population are shown in Table 1.

To analyze the systemic delivery of salbutamol by inhalation or oral application, we stimulated F508del/R117H and F508del/A455E organoids with plasma collected before and after *in vivo* treatment. Plasma collected after oral salbutamol treatment significantly induced F508del/R117H organoid swelling compared with the plasma collected before treatment or after aerosol administration of salbutamol, indicating that plasma concentrations of salbutamol were highest after oral treatment (Fig. 4a,b). Spiking of pure salbutamol in pooled plasma of subjects before treatment indicated that active salbutamol levels were below detection levels upon aerosol treatment and detectable but low after oral treatment, amounting to ~5 nM (Fig. 4c). After correcting for 40% subject plasma samples, circulating salbutamol levels after oral treatment on average reached levels ~12.5 nM.

To monitor *in vivo* modulation of CFTR function, SCC and NPD measurements were performed before and after 3 days of treatment with salbutamol. An overview of the changes in the SCC and NPD values is given in tables 2 and 3. Consistent with the outcome of the salbutamol bioassay showing low (oral treatment) or undetectable (aerosol treatment) levels of salbutamol in the plasma (nanomolar range), the only significant change in NPD was seen upon oral (but not aerosol) treatment, *i.e.* the median baseline potential changed significantly (by 6.4 mV) in the direction of reduced sodium absorption, indicative of an improved CFTR function (Table 2). However, we did not observe any significant changes in other NPD parameters nor in levels of sweat chloride (Tables 2 and 3 and Supplementary Tables S2 and S3).

Table 1. Baseline characteristics of the 10 subjects enrolled in the pilot study

Age years	38.5	(31.5-49.0)
Male	4	(40)
Body mass index kg·m^{-2}	22.28	(30.38-28.16)
FEV$_1$ % pred (range)	62.0	(44.8-84.8) (31-109)
CFTR genotype		
F508del/A455E	9	(90)
F508del/R117H	1	(10)

Data are presented as median (interquartile range) or n (%), unless otherwise stated. FEV1: forced expiratory volume in 1 s.

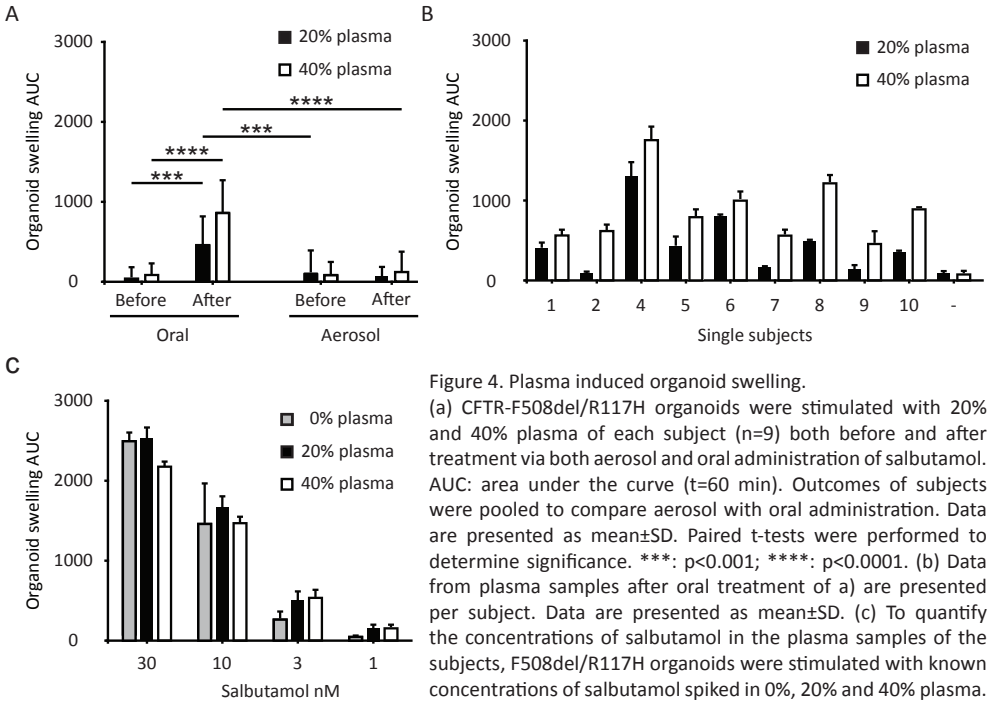

Figure 4. Plasma induced organoid swelling.
(a) CFTR-F508del/R117H organoids were stimulated with 20% and 40% plasma of each subject (n=9) both before and after treatment via both aerosol and oral administration of salbutamol. AUC: area under the curve (t=60 min). Outcomes of subjects were pooled to compare aerosol with oral administration. Data are presented as mean±SD. Paired t-tests were performed to determine significance. ***: p<0.001; ****: p<0.0001. (b) Data from plasma samples after oral treatment of a) are presented per subject. Data are presented as mean±SD. (c) To quantify the concentrations of salbutamol in the plasma samples of the subjects, F508del/R117H organoids were stimulated with known concentrations of salbutamol spiked in 0%, 20% and 40% plasma.

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Table 2. Sweat chloride and nasal potential difference (NPD) response to oral salbutamol

Parameter	Before oral treatment		After oral treatment		Change during oral treatment	p-value
Subjects	10		10			
Sweat chloride mmol·L^{-1}	72.5	(66.8-82.3)	73.0	(67.3-77.0)	+0.5	0.359
NPD measurement mV						
Basal potential	-55.2	(-62.0--43.4)	-48.8	(-57.5--38.9)	+6.4	0.047$^{\#}$
Δamiloride	38.4	(26.6-44.8)	38.8	(27.8-45.1)	+0.4	0.878
ΔCl$^-$ free	-1.4	(-6.7-5.9)	-5.6	(-10.0--1.2)	-4.2	0.203
Δisoproterenol	-1.4	(-2.7--2.9)	-0.1	(-2.9-1.7)	+1.3	0.646

Data are presented as n or median (interquartile range), unless otherwise stated. $^{\#}$: p<0.05.

Adverse events that were reported during treatment with salbutamol appeared higher for oral salbutamol and are summarised in table 4. The data of this pilot study tentatively indicate that oral, but not inhaled, treatment with β2-agonists may slightly improve residual CFTR function in nasal epithelium *in vivo*, but failed to further improve CFTR function in the sweat duct.

Table 3. Sweat chloride and nasal potential difference (NPD) response to salbutamol aerosol

Parameter	Before oral treatment		After oral treatment		Change during oral treatment	p-value
Subjects	9		9[#]			
Sweat chloride mmol·L^{-1}	67.0	(58.5-71.0)	70.0	(62.5-73.0)	+3.0	0.479
NPD measurement mV						
Basal potential	-47.3	(-54.4--40.9)	-41.6	(-49.8--37.4)	+5.7	0.123
Δamiloride	29.6	(23.4-41.7)	29.0	(19.8-36.6)	-0.6	0.208
ΔCl$^-$ free	4.2	(-8.5-3.8)	-1.6	(-8.1--0.2)	+2.6	0.401
Δisoproterenol	-2.4	(-4.6--0.9)	-2.0	(-4.7--0.7)	+0.4	0.878

Data are presented as n or median (interquartile range), unless otherwise stated. [#]: n=8 for NPD measurement after aerosol treatment.

Table 4. Adverse events during treatment with salbutamol

Adverse event	During oral treatment	During aerosol treatment
Agitated feeling	1	
Palpitations	4	
Cough up more sputum	2	
Dry mouth	1	1
Tremor	5	1
Headache	1	1
Painful breathing		1

Data are presented as n times reported.

Discussion

The purpose of this study was to generate proof-of-concept that organoid-based measurements can be used to identify approved drugs that may modulate CFTR function *in vivo*. In this study, CFTR function measurements in organoids were applied to (i) prioritise potential drugs out of multiple candidates, (ii) identify and verify subjects with potential responsive CFTR variants, and (iii) study the optimal route of administration of the drug. As a whole, the results of the pre-clinical and clinical studies together indicate that organoid-based measurement can aid in designing clinical studies for subjects with CF.

We selected β_2-agonists from 61 compounds that can modulate GPCR signalling, which are known activators of CFTR and anion transport. Surprisingly, the potency of β_2-agonists to stimulate CFTR function was equal or even better than forskolin, which directly stimulates adenylyl cyclase downstream of GPCR, as observed in both primary intestinal and

airway cells (Fig. 2 and 3) [13,29]. The formation of macromolecular complexes between β2-adrenergic receptors and CFTR may enable this efficient coupling of signals from β2-adrenergic receptors to CFTR [30]. The lack of CFTR activation by other compounds in this library most likely reflects the absence of their cognate receptors or their inability to induce sufficient CFTR activating signals or coupling these to CFTR.

Measurement of *in vivo* CFTR function enhanced by exogenous activators such as β_2-agonists requires a different approach as compared to direct CFTR-protein restoring drugs such as VX-770 and VX-809. It relies on the ability of exogenous β_2-agonists to phosphorylate and stimulate CFTR activity beyond levels associated with endogenous conditions. We anticipated that NPD could provide the most promising readout for an improvement of CFTR function, as intranasal infusion with the pan-β-agonist isoproterenol has been shown to further hyperpolarise the nasal epithelium in healthy controls *in vivo* by an average of 6.9 mV under low luminal chloride conditions [31-32], suggesting that CFTR activity in this tissue is rate-limited by endogenous cAMP signaling. Whereas for diagnostic purposes and direct CFTR protein-restoring drugs, the combined change in NPD after addition of zero chloride solution and addition of isoproterenol is most informative, in patients treated with salbutamol we anticipated to find an enhanced response to low chloride but a reduced response to intranasally applied isoproterenol and no difference in the combined response to low chloride plus isoproterenol as both compounds activate endogenous cAMP. Although there was a tendency to an enhanced hyperpolarising response to zero chloride (−1.4→−5.6 mV) and a decreased response to isoproterenol (−1.4→−0.1 mV) upon oral (but not inhaled) treatment, this difference did not reach statistical significance. The baseline potential difference, which was clearly CF-like in both the A455E and the R117H patients (range −41.6–−55.2 mV; Tables 2 and 3) showed a significant but limited increase towards non-CF baseline potential difference values (by 6.4 mV; Table 2) in the orally treated patients, but this change was not paralleled by a reduced response to amiloride, an inhibitor of ENaC (Table 2). Baseline potential appears to be predominated by ENaC-dependent Na+ absorption, which is enhanced in CF and modulated through direct CFTR protein-targeting drugs [33-35]. This lack of correlation seems to argue against an inhibitory effect of salbutamol treatment on ENaC activity, although the relatively low power of the pilot study and the large variation in potential difference responsiveness to ENaC does not entirely rule out such an effect. The latter interpretation would be in line with the tentative increase in zero chloride response discussed above and the known inhibitory effect of CFTR (through electrogenic or more complex coupling mechanisms) on ENaC activity in the airways. Alternatively, our data do not exclude the possibility that salbutamol inhibits other electrogenic ion transport pathways in the nasal epithelium, such as apical cation channels different from ENaC, e.g. acid-sensitive ion channels [36] or proton channels[37]. Taken together, the outcome of the NPD measurements suggests that oral salbutamol treatment slightly modifies the electrical properties of the nasal CF epithelium towards that of the non-CF nasal epithelium and could therefore be of (limited) benefit for the CF patients

The lack of robust CFTR activation *in vivo*, in clear contrast with the potent stimulation of organoid swelling by submicromolar levels of salbutamol *in vitro* (Fig. 2d), is most likely due to the low levels of circulating β2-agonist observed upon in vivo treatment. In line with the findings in the NPD measurements, we only found a detectable CFTR-activating capacity in blood samples that were collected after treatment with oral, but not with inhaled salbutamol. The latter is consistent with a highly limited systemic delivery by β2-agonist

inhalation [38] (Fig. 4a). However, even after treatment with oral salbutamol, CFTR-activating levels in plasma were low (Fig. 4a,b), corresponding with pure salbutamol concentrations in the nanomolar range that can only marginally stimulate organoids (compare Fig. 4c and 2d). As such, only limited effects *in vivo* might have been expected, as we observed in only one of the NPD parameters. Although plasma levels are not similar to tissue levels, these data suggest that higher dosages may further improve efficacy of treatment, albeit that systemic side-effects of the treatment may limit the feasibility of increasing the dosage.

The lack of response in SCC is also indicative of a limited treatment response, albeit that this parameter needs to be interpreted with care. SCC is a highly sensitive CFTR function parameter, being capable of distinguishing between pancreas-sufficient and -insufficient groups, and pre-treatment SCC in our patient cohort clearly indicates that these patients have significant residual CFTR activity (Table 3). Furthermore, in G551D patients this biomarker is also highly responsive to CFTR potentiator treatment [10]. However, isolated sweat glands from subjects also indicate that exogenous β-adrenergic stimuli can only stimulate ~40% of sweat ducts and later studies confirmed a high constitutive cAMP-dependent activation of CFTR in this tissue [39-40]. This implies that only a limited window for exogenous β2-agonist stimulation likely exists *in vivo* in this tissue. The lack of treatment response in SCC we observed in this trial was therefore not completely unexpected considering the high constitutive CFTR activation in this tissue combined with the very low levels of circulating β2-agonists.

Additional clinical studies are required to further validate the effect of long-term treatment with an oral β2-agonists on clinical outcome parameters (e.g. percentage predicted forced expiratory volume in 1 s, airway resistance, body mass index, quality of life) in CF patients, as this proof-of-concept study showed a minor but significant impact of treatment on the nasal mucosa, but no significant effect on Cl^- transport in the sweat ducts.

As expected, β2-agonists stimulate swelling of organoids in a CFTR mutation-dependent manner, based on residual function conferred by the CFTR genotype or by CFTR-modulating drugs (Fig. 2). Most subjects included in the study were compound heterozygous for A455E, and their organoids demonstrate residual CFTR function levels between the values seen with F508del and R117H/F508del compound heterozygotes [20]. This appears consistent with the SCC parameters measured in this study and with data from the CFTR2 database (www.cftr2.org). Our NPD data (Tables 2 and 3 and Supplementary Tables S2 and S3: response to chloride-free and isoproterenol) also showed evidence of residual CFTR function in the A455E patients, with a tendency to increase slightly but not significantly after oral salbutamol treatment. As the VX-770+VX-809-corrected CFTR-F508del function in organoids is higher than the level of residual function associated with CFTR-A455E [20] (Fig.2), β2-agonists may also have added value for F508del homozygous subjects treated with CFTR-repairing drugs. In this context, cotreatments with β2-agonists may account for some of the pulmonary heterogeneity between patients that is observed in the response to CFTR modulator treatment [9,11]. In addition, further stratification for CFTR genotypes with higher residual function (e.g. CFTR-R117H) may also enhance treatment effects with β2-agonists.

In conclusion, CFTR function measurements in intestinal organoids were used to screen for CFTR-activating drugs and subjects with CFTR variants that respond to these drugs *in vitro* were selected for *in vivo* treatment. Oral treatment with salbutamol improved some CF characteristics of the nasal mucosa, but treatment efficacy was likely limited due

to ineffective dosage, as apparent from measurements of plasma levels of salbutamol in our organoid-based bioassay. The study supports the concept that intestinal organoids are a valuable tool for selecting drugs and route of administration for CF clinical trials.

Conflict of interest

JMB, CKE, JFD are inventors on a patent application related to these findings.

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Reference list

1 Riordan, J. R. CFTR function and prospects for therapy. *Annu Rev Biochem* **77**, 701-726, (2008).
2 Weiler, C. A. & Drumm, M. L. Genetic influences on cystic fibrosis lung disease severity. *Front Pharmacol* **4**, 40, (2013).
3 Vanscoy, L. L. *et al.* Heritability of lung disease severity in cystic fibrosis. *Am J Respir Crit Care Med* **175**, 1036-1043, (2007).
4 Sosnay, P. R. *et al.* Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene. *Nat Genet* **45**, 1160-1167, (2013).
5 Kerem, E. *et al.* The relation between genotype and phenotype in cystic fibrosis--analysis of the most common mutation (delta F508). *N Engl J Med* **323**, 1517-1522, (1990).
6 Castellani, C. *et al.* Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. *J Cyst Fibros* **7**, 179-196, (2008).
7 Zielenski, J. Genotype and phenotype in cystic fibrosis. *Respiration* **67**, 117-133, (2000).
8 Van Goor, F. *et al.* Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A* **106**, 18825-18830, (2009).
9 Ramsey, B. W. *et al.* A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *N Engl J Med* **365**, 1663-1672, (2011).
10 Accurso, F. J. *et al.* Effect of VX-770 in persons with cystic fibrosis and the G551D-CFTR mutation. *N Engl J Med* **363**, 1991-2003, (2010).
11 Wainwright, C. E. *et al.* Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N Engl J Med* **373**, 220-231, (2015).
12 Van Goor, F. *et al.* Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proc Natl Acad Sci U S A* **108**, 18843-18848, (2011).
13 Chan, H. C., Fong, S. K., So, S. C., Chung, Y. W. & Wong, P. Y. Stimulation of anion secretion by beta-adrenoceptors in the mouse endometrial epithelium. *J Physiol* **501 (Pt 3)**, 517-525, (1997).
14 Smith, J. J. & Welsh, M. J. cAMP stimulates bicarbonate secretion across normal, but not cystic fibrosis airway epithelia. *J Clin Invest* **89**, 1148-1153, (1992).

15 Waldman, D. B., Gardner, J. D., Zfass, A. M. & Makhlouf, G. M. Effects of vasoactive intestinal peptide, secretin, and related peptides on rat colonic transport and adenylate cyclase activity. *Gastroenterology* **73**, 518-523, (1977).
16 Jung, P. *et al.* Isolation and in vitro expansion of human colonic stem cells. *Nat Med* **17**, 1225-1227, (2011).
17 Sato, T. & Clevers, H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science* **340**, 1190-1194, (2013).
18 Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-265, (2009).
19 Dekkers, J. F., van der Ent, C. K. & Beekman, J. M. Novel opportunities for CFTR-targeting drug development using organoids. *Rare Dis* **1**, e27112, (2013).
20 Dekkers, J. F. *et al.* A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* **19**, 939-945, (2013).
21 Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762-1772, (2011).
22 de Lau, W. *et al.* Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature* **476**, 293-297, (2011).
23 Korinek, V. *et al.* Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**, 1784-1787, (1997).
24 Bruscia, E. *et al.* Isolation of CF cell lines corrected at DeltaF508-CFTR locus by SFHR-mediated targeting. *Gene Ther* **9**, 683-685, (2002).
25 Swiatecka-Urban, A. *et al.* Pseudomonas aeruginosa inhibits endocytic recycling of CFTR in polarized human airway epithelial cells. *Am J Physiol Cell Physiol* **290**, C862-872, (2006).
26 Vijftigschild, L. A., van der Ent, C. K. & Beekman, J. M. A novel fluorescent sensor for measurement of CFTR function by flow cytometry. *Cytometry A* **83**, 576-584, (2013).
27 Robert, R. *et al.* Correction of the Delta phe508 cystic fibrosis transmembrane conductance regulator trafficking defect by the bioavailable compound glafenine. *Mol Pharmacol* **77**, 922-930, (2010).
28 Dekkers, R. *et al.* A bioassay using intestinal organoids to measure CFTR modulators in human plasma. *J Cyst Fibros* **14**, 178-181, (2015).
29 Walker, L. C. *et al.* Relationship between airway ion transport and a mild pulmonary disease mutation in CFTR. *Am J Respir Crit Care Med* **155**, 1684-1689, (1997).
30 Naren, A. P. *et al.* A macromolecular complex of beta 2 adrenergic receptor, CFTR, and ezrin/radixin/moesin-binding phosphoprotein 50 is regulated by PKA. *Proc Natl Acad Sci U S A* **100**, 342-346, (2003).
31 Knowles, M. R., Paradiso, A. M. & Boucher, R. C. In vivo nasal potential difference: techniques and protocols for assessing efficacy of gene transfer in cystic fibrosis. *Hum Gene Ther* **6**, 445-455, (1995).
32 Boyle, M. P. *et al.* A multicenter study of the effect of solution temperature on nasal potential difference measurements. *Chest* **124**, 482-489, (2003).
33 Rowe, S. M. *et al.* Optimizing nasal potential difference analysis for CFTR modulator development: assessment of ivacaftor in CF subjects with the G551D-CFTR mutation. *PLoS One* **8**, e66955, (2013).
34 Knowles, M., Gatzy, J. & Boucher, R. Relative ion permeability of normal and cystic fibrosis nasal epithelium. *J Clin Invest* **71**, 1410-1417, (1983).
35 Knowles, M. R. *et al.* Abnormal ion permeation through cystic fibrosis respiratory epithelium. *Science* **221**, 1067-1070, (1983).
36 Qadri, Y. J., Rooj, A. K. & Fuller, C. M. ENaCs and ASICs as therapeutic targets. *Am J Physiol Cell Physiol* **302**, C943-965, (2012).
37 Iovannisci, D., Illek, B. & Fischer, H. Function of the HVCN1 proton channel in airway epithelia and a naturally occurring mutation, M91T. *J Gen Physiol* **136**, 35-46, (2010).
38 Halfhide, C., Evans, H. J. & Couriel, J. Inhaled bronchodilators for cystic fibrosis. *Cochrane Database Syst Rev*, CD003428, (2005).
39 Reddy, M. M. & Quinton, P. M. PKA mediates constitutive activation of CFTR in human sweat duct. *J Membr Biol* **231**, 65-78, (2009).
40 Reddy, M. M. & Quinton, P. M. cAMP activation of CF-affected Cl- conductance in both cell membranes of an absorptive epithelium. *J Membr Biol* **130**, 49-62, (1992).

Supplementary Table 1. Chemicals screened for their ability to induce CFTR function

Chemical name	CAS registry number
ADL5859 HCl	850173-95-4
Granisetron HCl	107007-99-8
SB939	929016-96-6
Carvedilol	72956-09-3
Ketanserin (Vulketan Gel)	74050-98-9
Domperidone (Motilium)	57808-66-9
Maprotiline hydrochloride	10347-81-6
Dehydroepiandrosterone(DHEA)	53-43-0
JTC-801	244218-51-7
Bisoprolol	104344-23-2
Naftopidil Dihydrochloride	57149-08-3
AM-1241	444912-48-5
Dapoxetine hydrochloride (Priligy)	129938-20-1
Loperamide hydrochloride	34552-83-5
Naphazoline hydrochloride (Naphcon)	550-99-2
WAY-100635	162760-96-5
Fingolimod (FTY720)	162359-56-0
Agomelatine	138112-76-2
Alfuzosin hydrochloride(Uroxatral)	81403-68-1
Dapagliflozin	461432-26-8
Enalapril maleate (Vasotec)	76095-16-4
Amfebutamone (Bupropion)	31677-93-7
Olanzapine (Zyprexa)	132539-06-1
Epinephrine bitartrate (Adrenalinium)	51-42-3
LY310762	192927-92-7
LY404039	635318-11-5
Dimebon (Latrepirdine)	97657-92-6
Nebivolol (Bystolic)	152520-56-4
Benserazide	14919-77-8
Oxymetazoline hydrochloride	2315-02-8
Dopamine hydrochloride (Inotropin)	62-31-7
BRL-15572	193611-72-2
Amisulpride	71675-85-9
Sumatriptan succinate	103628-48-4

Supplementary Table 1. Chemicals screened for their ability to induce CFTR function

Chemical name	CAS registry number
Silodosin (Rapaflo)	160970-54-7
Chlorpromazine (Sonazine)	69-09-0
Racecadotril (Acetorphan)	81110-73-8
Ritodrine hydrochloride (Yutopar)	23239-51-2
ADX-47273	851881-60-2
Asenapine	85650-56-2
Tianeptine sodium	30123-17-2
Risperidone (Risperdal)	106266-06-2
Clonidine hydrochloride (Catapres)	4205-91-8
Salbutamol sulfate (Albuterol)	51022-70-9
Clomipramine hydrochloride (Anafranil)	17321-77-6
BMY 7378	21102-95-4
Doxazosin mesylate	77883-43-3
Venlafaxine	99300-78-4
Quetiapine fumarate (Seroquel)	111974-72-2
Naftopidil (Flivas)	57149-07-2
Clozapine (Clozaril)	5786-21-0
Sotalol (Betapace)	959-24-0
Trazodone hydrochloride (Desyrel)	25332-39-2
Nepicastat hydrochloride	170151-24-3
Fluoxetine HCl	59333-67-4
Zibotentan (ZD4054)	186497-07-4
Betaxolol hydrochloride (Betoptic)	63659-19-8
LDE225 (NVP-LDE225)	956697-53-3
Pramipexole (Mirapex)	53-43-0
Xylazine (Rompun)	7361-61-7
RS-127445	199864-87-4

Supplementary Table 2. Sweat Chloride and Nasal Potential Difference response to oral salbutamol per subject included in the pilot study.

Subject	Sweat Chloride *(mmol/L)*		Nasal Potential Difference Measurement *(mV)*							
			Basal PD		Δ Amil		Δ Cl^- free		Δ Iso	
	Before treatment	After treatment	before treatment	after treatment	before treatment	after treatment	before treatment	after treatment	before treatment	after treatment
1	72	65	-20,5	-24,6	14,7	16,7	-4,0	-3,7	-0,4	7,1
2	82	87	-41,1	-40,5	31,4	29,9	3,7	8,4	-2,4	-4,5
3	83	77	-59,2	-50,5	42,9	45,8	-5,9	-7,0	-2,6	-1,0
4	53	45	-61,8	-55,2	33,9	41,8	5,3	-6,9	5,2	2,2
5	91	75	-45,0	-34,1	19,7	21,5	7,5	4,7	2,8	-1,2
6	75	76	-62,7	-58,7	46,5	44,9	1,2	-3,1	-1,6	1,5
7	67	71	-74,0	-65,4	54,9	48,4	8,7	-13,3	-3,5	-2,7
8	73	71	-52,2	-46,9	44,2	41,1	-12,3	-12,4	2,9	-3,5
9	72	68	-58,1	-57,1	43,3	36,6	-9,0	-4,4	-11,7	1,1
10	66	77	-44,1	-47,1	28,8	32,6	-4,1	-9,1	-1,2	0,9

Supplementary Table 3. Sweat Chloride and Nasal Potential Difference response to salbutamol aerosol per subject included in the pilot study.

Subject	Sweat Chloride *(mmol/L)*		Nasal Potential Difference Measurement *(mV)*							
			Basal PD		Δ Amil		Δ Cl^- free		Δ Iso	
	Before treatment	After treatment	before treatment	after treatment	before treatment	after treatment	before treatment	after treatment	before treatment	after treatment
1	67	75	-22,0	-19,7	13,2	16,0	-7,0	-0,5	4,6	-1,1
2	61	106	-43,7	-41,0	29,6	31,0	10,8	3,4	-1,2	-2,6
3	-	-	-	-	-	-	-	-	-	-
4	43	41	-38,1	-36,4	28,5	23,2	2,6	-4,6	-7,7	-0,6
5	67	70	-51,6	-42,2	18,2	18,7	5,0	0,5	-1,4	-4,2
6	79	71	-57,3	-51,8	47,9	37,5	-4,2	-2,5	-0,5	-1,3
7	74	69	-62,4	-71,1	44,6	44,4	-10,4	-9,3	-2,4	-4,8
8	68	70	-47,3	-43,9	38,9	34,1	-8,5	-13,3	-3,5	2,4
9	58	71	-50,8	-	29,5	-	-8,4	-	-2,5	-
10	59	56	-46,1	-40,7	32,0	27,0	-4,1	-0,6	-5,7	-5,2

6

Type I Interferon inhibits CFTR function by down regulation of CFTR expression in intestinal organoids

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Abstract

The autosomal recessive life-shortening disease cystic fibrosis (CF) is caused by mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene. *CFTR* encodes an apically expressed chloride channel, and dysfunctional CFTR impaires ion transport, and fluid homeostasis leading to viscous mucus, chronic infection and hyperinflammation of the pulmonary and gastrointestinal tract. Inflammatory cytokines have been found to modify CFTR function and their modulation may impact CF disease progression and response to novel CFTR modulators. However, a comprehensive analysis of the activity of cytokines on CFTR in primary CF cells is currently lacking. We aimed to identify inflammatory modulators that modulate CFTR function using a functional CFTR assay in intestinal organoids. After screening of 111 inflammatory modulators, we identified interferons (IFNs) to inhibit CFTR function in intestinal organoids by reducing CFTR mRNA and protein levels. We further explored regulatory pathways for interferon secretion in intestinal epithelium that might modulate CFTR function. Our data suggest that inhibition of IFN signaling in CF may lower CF disease progression by enhancing CFTR expression and function, especially for subjects with residual CFR function or upon treatment with CFTR-restoring drugs.

Introduction

Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene cause the autosomal recessive disease cystic fibrosis (CF) [1-4]. The most prevalent mutation is the deletion of phenylalanine at position 508 (F508del), with a frequency 66% of all mutated CF alleles [5-6]. The gene encodes an apically expressed chloride channel and loss-of-function gene mutations result in dehydration and acidification of mucus layers and accumulation of viscous mucus and pathogens in the lungs [7]. Bacterial colonization and chronic infection associated with excessive inflammation of the respiratory tract are the main contributors to pulmonary complications and reduced life expectancy of subjects with CF [8-10].

Treatment of CF is mostly symptomatic, including the use of antibiotics and mucolytic agents to clear the lungs from infections. Recently, small molecules like VX-809 [11] and VX-770 [12] have been developed to repair CFTR in a mutation-specific manner. The corrector VX-809 improves the folding and trafficking of CFTR-F508del to the plasma membrane thereby increasing expression and function. The potentiator VX-770 increases the open probability of apical-localized CFTR [13], including VX-809 corrected CFTR-F508del, and has been registered for approximately 5% of CF patients containing gating mutations. Recently, the combination of VX-809 and VX-770 was found effective for treatment of CFTR-F508del homozygous patients targeting approximately 50% of patients [14-15]. Despite these advances, the efficacy of these treatments remains insufficient to 'cure' CF and is variable between subjects. It is therefore highly important to identify novel mechanisms that control the efficacies of these novel CFTR-restoring treatments.

CF disease is marked by increased inflammation in the lungs and intestine [16-18]. The inflammatory response induced by pathogens and the thickened mucus is complex, and involves pattern recognition receptors on epithelial cells near the site of inflammation. Cytokines play an important role to shape the inflammatory response, and have also been described to modulate CFTR function. Tumor necrosis factor (TNF)-α and IFN-γ synergistically decrease the amount of CFTR mRNA, thereby affecting CFTR function in the intestinal epithelial cell lines T84 and HT-29 [19-20]. Furthermore, TGF-β1, the major plasma isoform of TGF-β, is also described to down regulate CFTR protein expression in epithelial cells lines *in vitro* and can reduce the efficacy of VX-809 treatment on CFTR-F508del [21]. In contrast, other cytokines are reported to increase the expression of CFTR, such as interleukin (IL)-1β [22], IL-4 [23] and IL-13 [24-25]. Most of these findings were obtained from transformed epithelial cell line cultures, which have lost essential characteristics of the *in vivo* tissue, and await replication in non-transformed primary tissue cultures. For the majority of cytokines, of which there are over 100 recognized now, it therefore remains unclear if they modulate CFTR expression and function, and how such modulation may impact the efficacy of CFTR-restoring treatments.

Here, we aimed to identify cytokines that modulate CFTR function in primary intestinal organoids using a recently developed functional CFTR assay [26]. Organoids are intestinal stem cell cultures, which are grown from isolated crypts of rectal biopsies. Organoids can be maintained long-term in culture, and consist of a single epithelial polarized cell layer that recapitulates the *in vivo* tissue architecture [27-29]. Raising intracellular cAMP levels with forskolin induces rapid fluid secretion into the enclosed lumen of the organoids in a fully CFTR-dependent fashion and causes rapid organoid swelling [26]. This swelling

assay was used to screen 111 different cytokines for their ability to modulate CFTR-F508del function in the presence of the CFTR modulator VX-770. Furthermore, we set out to identify inflammatory pathways by which CFTR-regulating cytokines can be modulated.

Methods and Marerials

Ethical statement - Approval for this study was obtained by the ethics committees of the University Medical Centre Utrecht and Erasmus MC Rotterdam. Informed consent was obtained from all subjects participating in the study. Rectal organoids from healthy controls and subjects with cystic fibrosis were generated from four rectal suction biopsies after intestinal current measurements were obtained (i) during standard cystic fibrosis care, (ii) for diagnostic purposes or (iii) during voluntary participation in studies approved by the University Medical Center Utrecht and Erasmus MC ethics committees.

Organoid culture - Organoids were obtained and maintained in culture as previously described [26]. Briefly, after crypt isolation from rectal biopsies, the organoids were cultures for at least 3 weeks prior to use in the hereafter described experiments. Organoids were expanded in 50% Matrigel (BD) immersed in growth media containing advanced DMEM/F12 (Invitrogen), penicillin/ streptomycin (Invitrogen), Glutamax, Hepes (Invitrogen), N2 and B27 supplement (Invitrogen), conditioned media (Wnt3a, Rspo-1 and Noggin), N-acetylcysteine (Sigma-Aldrich), Nicotinamide (Sigma-Aldrich), mEGF (Sigma-Aldrich), Gastrin, SB202190 (p38 inhibitor; Sigma-Aldrich) and A83-01 (TGF-β1R inhibitor; Tocris). Growth media was replaced every 2-3 days, organoids were passaged weekly.

Functional CFTR measurements - Organoids were plated in a pre-warmed flat-bottom 96 wells plate in droplets containing 50% matrigel surrounded with growth media. After overnight (20-28 h) incubation at 37°C, organoids were labeled with calcein AM (2.5 μM) for 15 to 30 min. Hereafter, organoids were stimulated by the addition of a cAMP agonist and swelling was monitored for 60 min using a ZEISS LSM 710.

Primary cytokine screen - Organoids, expressing CFTR-WT or CFTR-F508del, were plated as described above. The inhibitors, SB202190 and A83-01, were removed from the growth media during overnight cytokine stimulation. Cytokine concentrations were diluted (1:500, 1:2500, 1:12500 and 1:62500) from original stocks (see supplementary table 1 for detailed information on the used cytokines). After overnight cytokine incubation, the organoids were labeled with calcein AM and stimulated with forskolin (5 μM) and VX-770 (1 μM). Cytokine stimulated samples were single measurements, controls were measured in duplicate. To assess direct activity of cytokines on organoid swelling organoids from healthy individuals were labeled with calcein AM one day after plating. After labeling, the cytokines were added to the organoids and swelling was monitored for 60 min using a ZEISS LSM 710.

Quantification of organoids swelling - Total organoid surface area was measured per time point and the relative increase of surface area in time was used as measure for CFTR activity [26]. Volocity (PerkinElmer) was used for image analysis and area under curve was calculated in Microsoft Excel using four time points (0, 20, 40, 60 min).

Selection of positive hits for rescreening - Measurements were normalized to forskolin (5 μM) and VX-770 (1 μM) per 96 well plate. Cytokines selected for rescreening met one of the following criteria: (I) dose-dependent responses were all above or below 100%, with mean response at least 15% higher or lower than baseline, or (II) the responses are concentration-dependent with a difference of at least 20% between the highest and lowest cytokine concentration

RT-PCR - Organoids were harvested after 6-8 day culture, washed in PBS and lysed in TRIzol (Invitrogen). Chloroform was added and mixed. After centrifugation (13.000 x g for 15 min at 4°C), the transparent layer was transferred to a new tube and isopropanol was added at a 1:1 volume ratio. After 10 min incubation at RT the RNA was collected by centrifugation (13.000 x g for 15 min at 4°C). RNA was washed with 75% ethanol, air-dried and dissolved in RNase free water. For pathogen recognizing receptor mRNA detection, RNA was isolated using the RNeasy minikit (Qiagen), including a DNase treatment. RNA purity and quantity was assessed by spectrometry using a NanoDrop 2000 (Life Technologies). cDNA was synthesized by iScript Reverse Transcriptase (Bio-Rad) using 500 ng total RNA according to the manufacturer's specifications. SYBR Green (Bio-Rad) mix was used for PCR reaction, cDNA was diluted 1:25, final primer concentration was 0.5 μM in a total volume of 15 μL. The following PCR protocol was used, 3 minutes at 95°C followed by 40 cycles of denaturing (30 s at 95°C), annealing (30 s at 60°C) and extension (30 s at 72°C) steps. Finally, a melting curve was made by heating the samples to 95°C for 1 min, cooling to 65°C for

1 min, and increasing the temperature every 10 s by 0.5°C, while monitoring the fluorescence signal. Afterwards, the PCR products were analyzed by 2% agarose gel electrophoresis. The list of primers used can be found in supplementary table 2.

Western blot analysis - Organoids from a 6-8 day culture were harvested and washed with PBS and lysed in laemmli buffer (4% SDS, 20% glycerol and 120mM Tris pH 6.8 in H_2O). After homogenizing the sample, using a G25 syringe, the protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce). Proteins were separated by SDS-PAGE and transferred overnight to a polyvinylidene difluoride membrane. Ponceau S staining was performed to validate the protein transfer. After blocking the membrane in TBST (10mM Tris pH 8, 150mM NaCl, 0.3% Tween 20) solution containing 5% low-fat milk powder (ELK) for 1 h, the membrane was incubated with monoclonal antibody 450, 570 and 596 (all 1:5000 in TBST containing 1% ELK; antibodies were provided by the Cystic Fibrosis Foundation Therapeutics (CFFT) Antibody Distribution Program) for an hour. The membranes were washed three times for 10 min in TBST. Next, a secondary goat anti-mouse antibody labelled with HRP (1:5000 in TBST containing 1% ELK) was incubated for 30-45 min, followed by three 10 min washing steps with TBST, and three 10 min washings with PBS. Finally, the HRP label was detected by enhanced chemiluminescence (ECL) and high performance X-ray films (Amersham Biosciences).

Pattern recognition receptor stimulation. - Individual PPRs were stimulated overnight with PAM3CSK4 (1 μg/mL), Poly (I:C) (1 μg/mL), LPS (1 ng/mL), imiquimod (1 μg/mL), ODN M362 (1 μM) for TLR1, TRL3, TLR4, TLR7 and TLR9, respectively. Organoids were stimulated with forskolin and VX-770 and swelling was monitored for 60 min.

Results:

Optimizing and validating intestinal organoid cultures for cytokine screening

Our aim was to identify cytokines that modulate CFTR function. Since the organoid growth media contains the p38 mitogen-activated protein kinase inhibitor (SB202190) and TGF-β1R inhibitor (A83-01) that are involved in cytokine signaling [30-31], we first investigated the effect of removal of these inhibitors on forskolin induced swelling (FIS) of organoids. As demonstrated previously, FIS of F508del homozygous organoids was found to be increased by the CFTR modulators treatment (organoids were stimulated with single agents (VX-770, VX-809), or their combination) (Fig. 1A). Removal of the inhibitors combined with VX-770 or VX-809 correction of CFTR-F508del function, markedly increased forskolin induced swelling (FIS) (Fig. 1B, representative images of VX-770 stimulated conditions are indicated in Fig. 1C). Removal of the p38 inhibitor increased the FIS of VX-770 treated CFTR-F508del more clearly as compared to withdrawal of the TGF-β1R inhibitor. Overnight removal of both inhibitors in combination with VX-770 potentiation was almost as effective as VX-770/VX-809 combination treatment. In non-treated CFTR-F508del organoids, we could not observe increased swelling upon removal of the p38 and TGF-β1R inhibitors. Removal of the inhibitors in VX-809/VX-770 treated organoids had limited effect, suggesting loss of dynamic range of the assay at these higher levels of CFTR function, as previously observed [26].

Inhibition of p38 and TGF-β1R is essential for long-term growth of organoids, and removal of these inhibitors potentially causes differentiation of the organoids from a crypt cell phenotype towards a more villus cell phenotype [28]. To establish if the organoids underwent terminal differentiation, we quantitated the level of intestinal stem cells in the organoids by measurement of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) mRNA. Overnight removal of the inhibitors only slightly changed LGR5 mRNA levels, which was in sharp contrast with transfer of organoids to differentiation media for five days. This suggested that overnight removal of the inhibitors did not significantly impact the differentiation of the organoids, and suggested that enhanced detection of swelling may

have been directly related to regulation of CFTR expression.

Identification of CFTR function modulating cytokines

To validated the expression of common cytokine receptor signaling subunits on intestinal organoids, RT-PCR was performed to investigate the presence of GP130, common-β and -γ chain mRNA in organoids. PBMC were used as positive control. Both GP130 and common-γ chain could be detected (Fig. 2A) whereas expression of the common-β chain (that signals via IL-3, IL-5 and GM-CSF) could not be validated. This suggests that a large number of cytokines may potentially modify intestinal organoid function when their cytokine-specific alpha chains are also co-expressed. To establish which cytokines modulate CFTR-F508del function, serial dilutions of 111 cytokines were independently incubated overnight with CFTR-F508del intestinal organoids in medium without the p38 and TGF-β1R inhibitors. FIS was examined in the presence of VX-770 to further potentiate CFTR-F508del function and facilitate that both stimulatory or inhibitory effects could be detected. Fourteen cytokines were identified that modulated CFTR function based on criteria described in details in the methods and materials section (consistently up or down regulated swelling phenotype, or a dose-dependent effect). Some cytokines were selected based on their stimulatory effect on CFTR-F508del mediated fluid secretion (VEGF, cathepsin B and cathepsin S), other cytokines appeared to inhibit CFTR function (FAS and IL-1RI), or were found to dose dependently modulate CFTR function (IL-21, IFN-α, IFN-β, IFN-γ, OSM, OPN, IL-8, adiponectin and IL-18BPa). Only IFN-α, -β and –γ were found to reproducible demonstrate a dose dependent inhibitory effect on CFTR function from the initial selection of 14 hits identified in the primary screen (Fig. 2B)

A second primary screen was set up to identify cytokines that activate CFTR, and consisted of the stimulation of organoids expressing CFTR-WT with cytokines without a cAMP agonist. Out of the initial 111 cytokines, 55 were selected that encompassed a representative subset of cytokines that could either stimulate via GP130 and the common γ-subunit, including IL-6/11/12/27 which are known cytokines utilizing the GP130 receptor and IL-2/4/7/9/13/15 which use the common γ-subunit (see Supplementary Table 3). None of these modulators were found to directly induce fluid secretion.

Mechanism of IFN-dependent CFTR modulation

To further study the role of IFN-dependent modulation of CFTR function, we examined expression of IFN receptors and CFTR expression levels after IFN treatment. IFN-α/β receptor (IFN-αβR) and IFN-γ receptor (IFN-γR) mRNA expression in organoids was analyzed by RT-PCR and their presence was detected supporting a direct stimulatory role for the IFNs (Fig. 3A). Quantitative RT-PCR indicated that overnight IFN-α, -β or -γ stimulation reduced CFTR mRNA levels (Fig. 3B). Reduced levels of CFTR protein accompanied the reduced CFTR mRNA levels as indicated by Western blot (Fig. 3C). These data suggest that IFN signaling inhibits CFTR gene expression, resulting in lower CFTR protein and function.

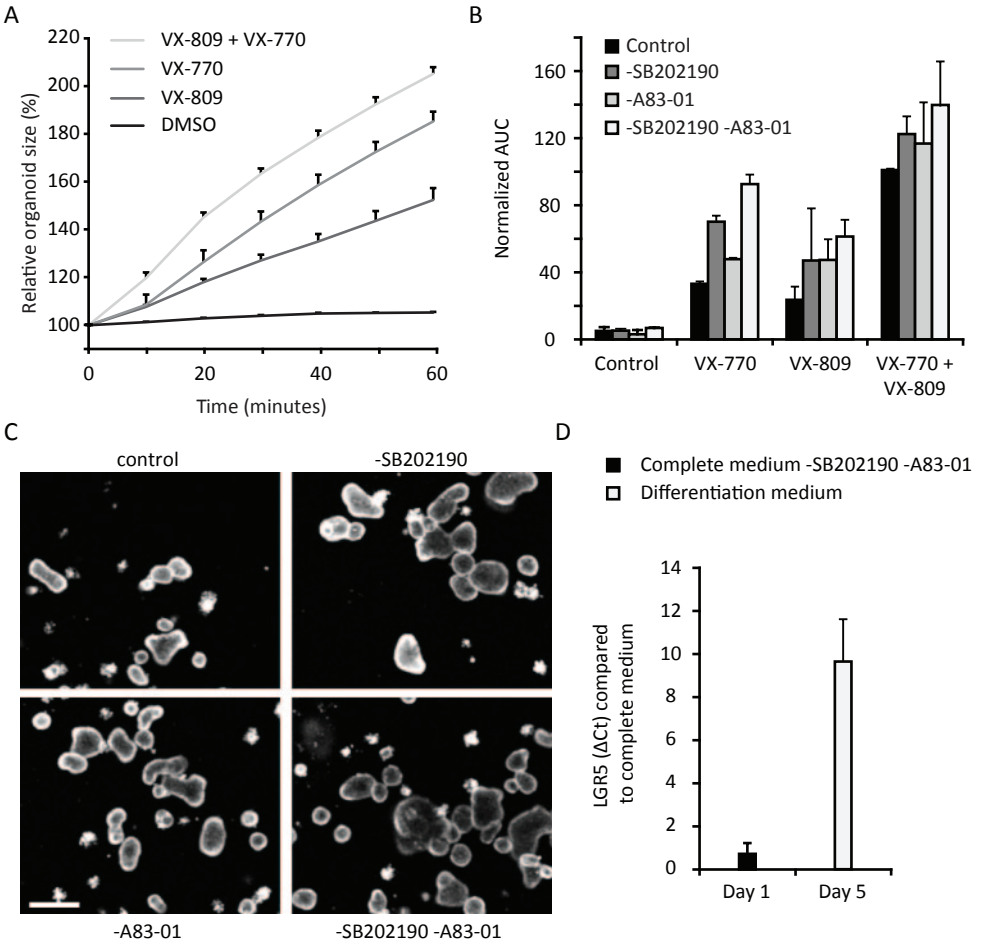

Figure 1. P38 and TGF-β modulation influences VX-770 and VX-809 correction of CFTR-F508del.
(a) Relative F508del organoids swelling after stimulation with forskolin (5 µM) and VX-770 (1 µM) (where indicated), after overnight removal of p38 (SB202190) and TGF-βR1 (A83-01) inhibitors. Where indicated, VX-809 (3 µM) was incubated overnight with the organoids. Representative experiment measured in triplicate, data presented as mean ± SD. (b) Quantification of CFTR-F508del function upon removal of SB202190 and A83-01 inhibitors, from overnight culture of organoids in combination with different CFTR restoring drugs. VX-809 (3 µM) was incubated overnight, VX-770 (1 µM) was added together with forskolin (5 µM). Data from 2 experiments measured in triplicate, presented as mean ± SD. (c) Representative confocal images of F508del/F508del organoids after 60 min stimulation with forskolin (5 µM) and VX-770 (1 µM), after overnight removal of SB202190 and A83-01 inhibitors. (d) qRT-PCR on, the intestinal stem cell marker, LGR5 performed on organoids cultured in complete medium, upon removal of SB202190 and A83-01 inhibitors for 1 day or after 5 days of culture in differentiation medium (complete medium without Wnt3a, nicotinamide, SB202190 and A83-01 [28]). Data of a single experiment, represented as ΔCt values compared to complete culture medium (mean±SD).

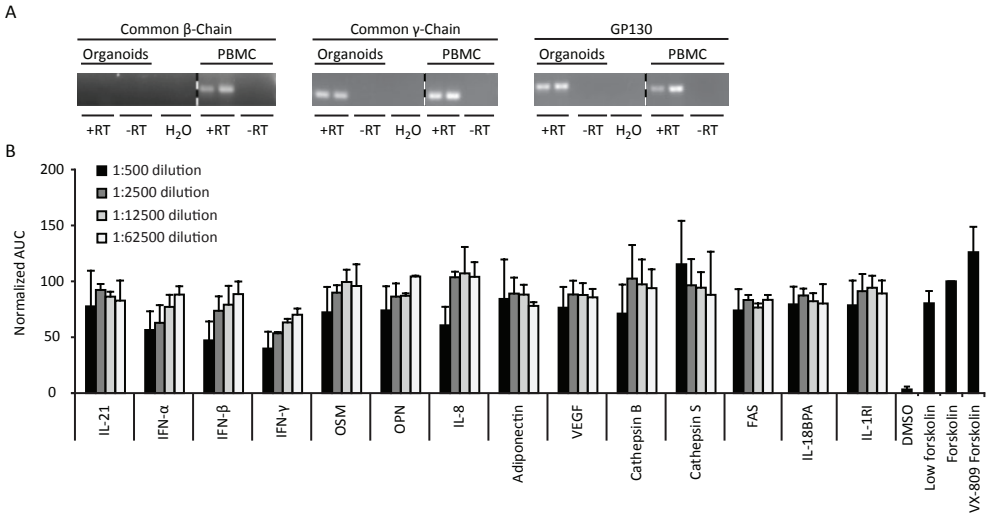

Figure 2. Presence of common signaling receptors on organoids and cytokines that potentially modulate CFTR function.

(a) RT-PCR on common β-, γ-chain and GP130 were performed in quadruplicate, as negative controls no reverse transcriptase (-RT) and H_2O were used. mRNA isolated from PBMCs were used as positive controls. Samples were analyzed on the same gel, dotted line indicates were lanes were removed. (b) Cytokines which were selected from primary screen. Cytokines were incubated with organoids overnight in 4 different concentrations and stimulated with forskolin (5 μM) and VX-770 (1 μM). Data presented as mean (± SD) from 3 independent experiments. Data are normalized to forskolin response.

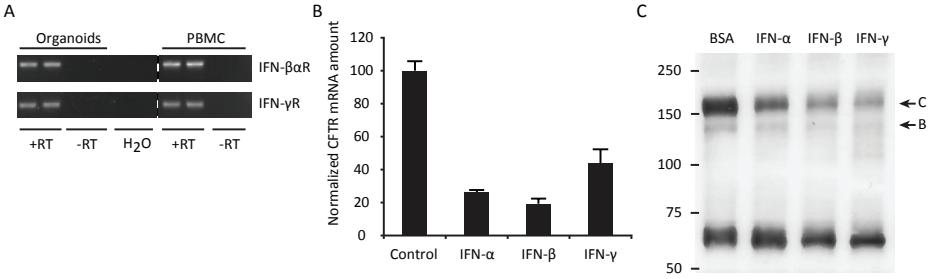

Figure 3. Interferon mediated CFTR down regulation.

(a) RT-PCR on IFN-βαR and IFN-γR from CF organoids was performed in quadruplicate, H_2O and omission of reverse transcriptase (-RT) were used as negative controls, mRNA derived from PBMCs was used as positive control. Samples were analyzed on the same gel, dotted line indicates were lanes were removed. (b) CFTR mRNA analysis upon overnight incubation with indicated cytokine (used at 1:500 dilution). CFTR mRNA was normalized to B2M expression using the ΔΔCT method [32]. Data from 3 independent experiments and presented as mean ± SD. (c) CFTR-WT organoids were incubated overnight with the IFN-α (10ng/mL), IFN-β (0.4ng/mL), IFN-γ (5ng/mL) or PBS-BSA (control) and CFTR expression was analyzed by SDS-PAGE and Western blotting. CFTR B- and C-band are indicated in the figure.

Characterizing regulatory pathways for intestinal IFN production and CFTR modulation

We aimed to investigate upstream inflammatory pathways that may lead to intestinal epithelial type I IFN production that presumably could be part of an innate defense response of the epithelium upon pathogen challenge. Our first goal was to study expression

of pattern recognition receptors (PRR) by organoids that may potentially drive production of IFN. By RT-PCR, we detected TLR1, -2, -3, -4, -5, -9, RIG-I and MDA-5, whereas TLR-6, -7, -8 and -10 were not detected (Fig. 4). Peripheral blood mononuclear cells served as positive control, and suggested expression of all the PRR. These results indicate that organoids express many different PRRs, of which some may be involved in regulating epithelial type I interferon and CFTR. We next stimulated organoids directly with various TLR ligands that are known to activate specific TLRs. In a first experiment, we observed no significant differences in FIS of CFTR-F508del organoids after overnight PRR ligand incubation (Fig. 5). Together, these preliminary data did not provide indications that PRR could potentially regulate CFTR function.

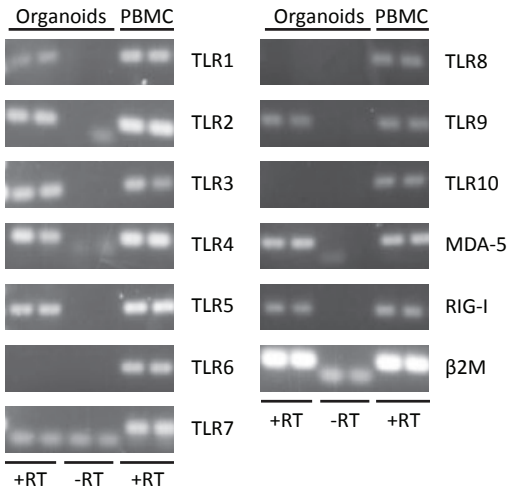

Figure 4. Presence of pattern recognition receptors mRNA in organoids detected by RT-PCR.
mRNA was isolated from organoids and the presence of different Toll like receptors was analyzed. Absence of reverse transcriptase (-) during cDNA synthesis and water were used as negative control, mRNA isolated from PBMCs were used as positive controls, however no DNase treatment was performed on PBMCs. DNase treatment was performed on mRNA isolated from organoid samples. A representative experiment out of three is shown.

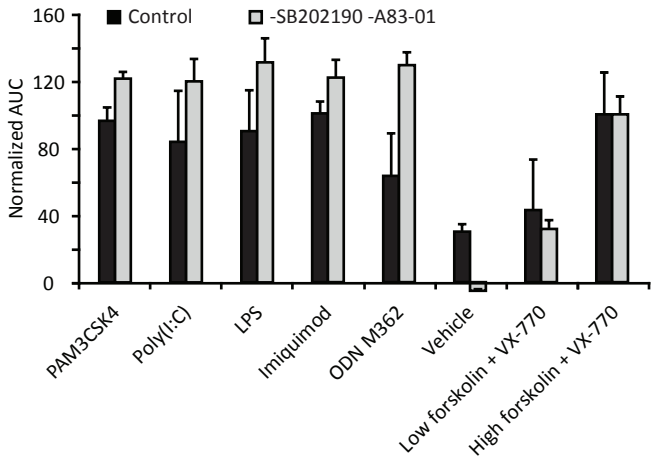

Figure 5. CFTR activation after overnight stimulation of pattern recognition receptor stimulation in organoids.
After overnight stimulation of PPR, organoids were stimulated with VX-770 (1 μM) and low forskolin (0.1 μM) or high forskolin (5 μM). Data was normalized to VX-770 forskolin response, single experiment measured in triplicate, mean ±SD.

Discussion

The aim of this study was to identify inflammatory mediators that modulate CFTR function using a primary intestinal cell model. A first goal was to set up a screening condition that would allow us to identify inflammatory mediators that could either activate or inhibit CFTR function. We first removed the p38 and TGF-β1R inhibitors from the assay media, as the presence of these regulators of cytokine signaling may confound the results of screening. Their removal consistently enhanced CFTR-F508del function, which was more prominent for the p38 inhibitor. These data validates earlier observations concerning the role of p38 signaling in modulating CFTR function, and show for the first time that p38 signaling is important to enhance CFTR function in primary intestinal epithelium. This may involve the post-transcriptional regulation of CFTR mRNA stability as observed in intestinal epithelial cell lines [20]. Our data also indicated that TGF-β signaling increased CFTR function, which contrasts with data from human carcinoma cell lines and primary epithelial cells lining the vas deferens [33-34]. This apparent difference may be caused by the multifaceted way of TGF-β1R signaling that can involve both SMAD dependent or independent routes in a tissue-specific fashion [35]. The removal of the p38 and TGF-β1R inhibitors ensured that the assay readout could by further enhanced or decreased in conditions when F508del-CFTR organoids were stimulated with only forskolin and VX-770. We preferred the use of VX-770 over VX-809 as this is added together with forskolin and directly potentiates the apical CFTR, thereby limiting potential interactions between cytokines and effects of VX-809 that is pre-incubated with organoids. In total 111 inflammatory mediators were tested for their ability to modulate CFTR-F508del function in organoids. For the large majority (108), we could not identify CFTR modulating activity. Potential explanations for this unexpected high number are: 1) only a few inflammatory modulators may impact CFTR function in primary intestinal cells, 2) the correct receptors may not be expressed at the basolateral membrane, 3) modulator concentrations may have been inadequate, 4) a lack of biological activity of the screened modulators , and 5) false negative results due to technical aspects related to the screening. It is difficult using the limited data available to weight these different options. Recent data indicate that effects of VX809 and VX770 as combination can be more clearly observed at lower forskolin concentrations [36], suggesting that the used assay conditions had only limited capacity to detect cytokines that could enhance organoid swelling, potentially leading to false negatives (point 5). In any case, the setup of the assay was sufficient to identify IFN-α, IFN-β as novel modulators of CFTR-F508del function, and we verified IFN-γ as regulator of CFTR function.
Mechanistically, we observed that IFNs reduced CFTR mRNA levels, which was associated with reduced CFTR protein levels and function of VX-770 stimulated CFTR-F508del. This may result from mRNA destabilization rather than gene expression as this was found the primary mechanisms for IFN- γ induced CFTR mRNA down regulation in HT-29 and T84 cells [20]. Despite IFN-α, IFN-β and IFN-γ signal via different receptors and can induce distinct cellular effects [37], all three cytokines can also lead to the formation of active STAT1 homodimers. Indeed, earlier data indicate that IFN-γ down regulation of CFTR is STAT1 dependent in T84 cells, but these studies failed to demonstrate effect of IFN-α, and -β [38]. It is thus possible that STAT1 is responsible for the decreased expression of CFTR observed after stimulation with either IFN-α, -β or -γ in organoids, but further mechanistic studies are required to validate that a common IFN-induced signaling pathways is driving the IFN induced CFTR down regulation.

Type I and II interferons are associated with the innate immunity against bacterial and viral pathogens, of which type I interferons can be expressed by epithelial cells and act in auto- and paracrine fashions. Type I interferons can be directly induced by various PRRs [39], and we confirmed mRNA expression of the PRRs TLR3, TLR4, TLR9, RIG1 and MDA5 that have previously been implicated in upregulation of IFN type I. Epithelial innate defense may include the transient down modulation of CFTR function in healthy controls that may (partially) lead to typical viscous mucus production associated with infections that may limit spreading of infections. However, ligands for PRRs could not modulate CFTR swelling in preliminary experiments. The biological relevance of IFN-mediated CFTR down modulation in healthy controls remains unclear for now.

In cystic fibrosis subjects, some studies investigate the link between interferons and disease severity. IFN-β was detected in bronchial alveolar lavage fluid during exacerbations [40], which also correlates with a decrease in pulmonary function. In contrast, IFN-γ has been associated with stable CF disease, whilst being absent in acute exacerbations [41]. It thus indicates that type I and II interferons are part of the inflammatory phenotype in CF, albeit that the differential expression during active and stable disease may indicate that down regulation of CFTR is not a major contributor to disease severity. It is important to note that these studies have been conducted in subjects with severe CF who most likely lack significant residual CFTR function indicating that further down regulation of CFTR expression would not further reduce CFTR function. However, the impact of interferons on CFTR expression and CF disease development may be different in the context of CFTR mutations associated with CFTR residual function, or when CFTR protein restoring treatments are used. Current studies with CFTR modulators indicate that a homozygous subjects with F508del mutations respond differently to treatment than F508del heterozygotes suggesting that two-fold expression differences may impact therapeutic efficacy [15,36]. Furthermore, considerable heterogeneity in treatment between individuals with identical mutations are observed, and differences in activity of IFNs between patients may be one of the factors that contribute to such heterogeneity. It is therefore important to further study the link between CF disease and IFNs, especially in the context of CFTR modulator treatment or genetically encoded CFTR residual function.

In recent years, monoclonal antibodies have been developed to inhibit IFNs (for IFN-α and -γ), primarily to control inflammatory disorders such as systemic lupus erythematosus [42], chronic psoriasis [43], Crohn's disease [44] and rheumatoid arthritis [45]. However, repurposing these IFN-inhibiting antibodies for CF requires careful considerations, especially in a chronic setting, since this may be associated with a negative impact on CFTR-independent immune effector mechanism. This may lead to enhanced pathogen load and inflammation in this already infection-prone disease. Further, Th2-skewed adaptive immune response to fungi in patients with allergic bronchopulmonary aspergillosis have been associated with further disease aggravation, suggesting that the development of Th2 reactivity should be prevented. In this light, inhibition of type I interferons may be preferred over IFN-γ which is a critical cytokine for skewing towards a Th1 immune responses. To explore whether IFN inhibition may be helpful for CF, it may be investigated whether the short term inhibition of type I IFNs could induce CFTR function in patients with CFTR residual function.

In conclusion, we have used intestinal organoids to study the potential modulator of CFTR by 111 immune mediators. We showed that intestinal organoids express receptors required for cytokine signaling and responses to pathogens, and found that IFN-α, -β and –γ can

inhibit CFTR function by reducing CFTR mRNA expression. The data suggests that therapeutic interventions in CF aiming to modulate IFN activity may impact CFTR function, and could be particularly relevant in the context of CFTR residual function or CFTR modulator treatment.

Reference list

1 Riordan, J. R. CFTR function and prospects for therapy. *Annu Rev Biochem* **77**, 701-726, (2008).
2 Riordan, J. R. *et al.* Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066-1073, (1989).
3 Rommens, J. M. *et al.* Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* **245**, 1059-1065, (1989).
4 Kerem, B. *et al.* Identification of the cystic fibrosis gene: genetic analysis. *Science* **245**, 1073-1080, (1989).
5 Bobadilla, J. L., Macek, M., Jr., Fine, J. P. & Farrell, P. M. Cystic fibrosis: a worldwide analysis of CFTR mutations--correlation with incidence data and application to screening. *Hum Mutat* **19**, 575-606, (2002).
6 Kerem, E. *et al.* The relation between genotype and phenotype in cystic fibrosis--analysis of the most common mutation (delta F508). *N Engl J Med* **323**, 1517-1522, (1990).
7 Boucher, R. C. Cystic fibrosis: a disease of vulnerability to airway surface dehydration. *Trends Mol Med* **13**, 231-240, (2007).
8 Ratjen, F. & Doring, G. Cystic fibrosis. *Lancet* **361**, 681-689, (2003).
9 O'Sullivan, B. P. & Freedman, S. D. Cystic fibrosis. *Lancet* **373**, 1891-1904, (2009).
10 Rowe, S. M., Miller, S. & Sorscher, E. J. Cystic fibrosis. *N Engl J Med* **352**, 1992-2001, (2005).
11 Van Goor, F. *et al.* Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proc Natl Acad Sci U S A* **108**, 18843-18848, (2011).
12 Van Goor, F. *et al.* Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A* **106**, 18825-18830, (2009).
13 Van Goor, F., Yu, H., Burton, B. & Hoffman, B. J. Effect of ivacaftor on CFTR forms with missense mutations associated with defects in protein processing or function. *J Cyst Fibros* **13**, 29-36, (2014).
14 Boyle, M. P. *et al.* A CFTR corrector (lumacaftor) and a CFTR potentiator (ivacaftor) for treatment of patients with cystic fibrosis who have a phe508del CFTR mutation: a phase 2 randomised controlled trial. *Lancet Respir Med* **2**, 527-538, (2014).
15 Wainwright, C. E. *et al.* Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N Engl J Med* **373**, 220-231, (2015).
16 Pillarisetti, N. *et al.* Infection, inflammation, and lung function decline in infants with cystic fibrosis. *Am J Respir Crit Care Med* **184**, 75-81, (2011).
17 Ehre, C., Ridley, C. & Thornton, D. J. Cystic fibrosis: an inherited disease affecting mucin-producing organs. *Int J Biochem Cell Biol* **52**, 136-145, (2014).
18 Munck, A. Cystic fibrosis: evidence for gut inflammation. *Int J Biochem Cell Biol* **52**, 180-183, (2014).
19 Fish, S. M., Proujansky, R. & Reenstra, W. W. Synergistic effects of interferon gamma and tumour necrosis factor alpha on T84 cell function. *Gut* **45**, 191-198, (1999).
20 Besancon, F. *et al.* Interferon-gamma downregulates CFTR gene expression in epithelial cells. *Am J Physiol* **267**, C1398-1404, (1994).
21 Snodgrass, S. M., Cihil, K. M., Cornuet, P. K., Myerburg, M. M. & Swiatecka-Urban, A. Tgf-beta1 inhibits Cftr biogenesis and prevents functional rescue of DeltaF508-Cftr in primary differentiated human bronchial epithelial cells. *PLoS One* **8**, e63167, (2013).
22 Cafferata, E. G., Gonzalez-Guerrico, A. M., Giordano, L., Pivetta, O. H. & Santa-Coloma, T. A. Interleukin-1beta regulates CFTR expression in human intestinal T84 cells. *Biochim Biophys Acta* **1500**, 241-248, (2000).

23 Galietta, L. J. *et al.* IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro. *J Immunol* **168**, 839-845, (2002).
24 Skowron-zwarg, M. *et al.* Interleukin-13 interferes with CFTR and AQP5 expression and localization during human airway epithelial cell differentiation. *Exp Cell Res* **313**, 2695-2702, (2007).
25 Wu, D. *et al.* Interleukin-13 (IL-13)/IL-13 receptor alpha1 (IL-13Ralpha1) signaling regulates intestinal epithelial cystic fibrosis transmembrane conductance regulator channel-dependent Cl- secretion. *J Biol Chem* **286**, 13357-13369, (2011).
26 Dekkers, J. F. *et al.* A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* **19**, 939-945, (2013).
27 Sato, T. *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-265, (2009).
28 Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762-1772, (2011).
29 Jung, P. *et al.* Isolation and in vitro expansion of human colonic stem cells. *Nat Med* **17**, 1225-1227, (2011).
30 Ono, K. & Han, J. The p38 signal transduction pathway: activation and function. *Cell Signal* **12**, 1-13, (2000).
31 Schiffer, M., von Gersdorff, G., Bitzer, M., Susztak, K. & Bottinger, E. P. Smad proteins and transforming growth factor-beta signaling. *Kidney Int Suppl* **77**, S45-52, (2000).
32 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408, (2001).
33 Yi, S., Pierucci-Alves, F. & Schultz, B. D. Transforming growth factor-beta1 impairs CFTR-mediated anion secretion across cultured porcine vas deferens epithelial monolayer via the p38 MAPK pathway. *Am J Physiol Cell Physiol* **305**, C867-876, (2013).
34 Howe, K. L., Wang, A., Hunter, M. M., Stanton, B. A. & McKay, D. M. TGFbeta down-regulation of the CFTR: a means to limit epithelial chloride secretion. *Exp Cell Res* **298**, 473-484, (2004).
35 Derynck, R. & Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-584, (2003).
36 Dekkers, J. F. *et al.* Characterizing responses to CFTR-modulating drugs using rectal organoids derived from subjects with cystic fibrosis. *Sci Transl Med* **8**, 344ra384, (2016).
37 Platanias, L. C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5**, 375-386, (2005).
38 Kulka, M., Dery, R., Nahirney, D., Duszyk, M. & Befus, A. D. Differential regulation of cystic fibrosis transmembrane conductance regulator by interferon gamma in mast cells and epithelial cells. *J Pharmacol Exp Ther* **315**, 563-570, (2005).
39 Coccia, E. M. & Battistini, A. Early IFN type I response: Learning from microbial evasion strategies. *Semin Immunol* **27**, 85-101, (2015).
40 Kieninger, E. *et al.* High rhinovirus burden in lower airways of children with cystic fibrosis. *Chest* **143**, 782-790, (2013).
41 Wojnarowski, C. *et al.* Cytokine expression in bronchial biopsies of cystic fibrosis patients with and without acute exacerbation. *Eur Respir J* **14**, 1136-1144, (1999).
42 Petri, M. *et al.* Sifalimumab, a human anti-interferon-alpha monoclonal antibody, in systemic lupus erythematosus: a phase I randomized, controlled, dose-escalation study. *Arthritis Rheum* **65**, 1011-1021, (2013).
43 Bissonnette, R. *et al.* A randomized, double-blind, placebo-controlled, phase I study of MEDI-545, an anti-interferon-alfa monoclonal antibody, in subjects with chronic psoriasis. *J Am Acad Dermatol* **62**, 427-436, (2010).
44 Reinisch, W. *et al.* Fontolizumab in moderate to severe Crohn's disease: a phase 2, randomized, double-blind, placebo-controlled, multiple-dose study. *Inflamm Bowel Dis* **16**, 233-242, (2010).
45 Sigidin, Y. A., Loukina, G. V., Skurkovich, B. & Skurkovich, S. Randomized, double-blind trial of anti-interferon-gamma antibodies in rheumatoid arthritis. *Scand J Rheumatol* **30**, 203-207, (2001).

Supplementary Table 1. List of primers used for qRT-PCR

Target gene	Primer sequence
β2M	ATGAGTATGCCTGCCGTGTGA GGCATCTTCAAACCTCCATG
CFTR	ACCTGTCAACACTGCGCTGGT TCGGCTCACAGATCGCATCAAGC
Common γ-chain	CCAATGGGAATGAAGACACC GCAGAGTGAGGTTGGTAGGC
Common β-Chain	GAGCCAGTGTCCTGTGACCT TGGTCCTGGTCGGTGCTGAT
GP130	CATGCTTTGGGTGGAATGGAC CATCAACAGGAAGTTGGTCCC
IFN-αβR	TTGCTCTCCCGTTTGTCATTTA GACCTCAGGCTCCCAGTGTAAC
IFN-γR	TCCTCGATTGTCTTCGGTATGC GTGCTTAGCCTGGTATTCATCTGT
LGR5	GAATCCCCTGCCCAGTCTC ATTGAAGGCTTCGCAAATTCT
ABST	TGGCCCCAAAAAGCAAA AACCGTTCGGCACCTGTAC
TLR-2	GAATCCTCCAATCAGGCTTCTCT GCCCTGAGGGAATGGAGTTTA
TLR-4	GGCATGCCTGTGCTGAGTT CTGCTACAACAGATACTACAAGCACACT
TLR-5	TCAAACCCCTTCAGAGAATCCC TTGGAGTTGAGGCTTAGTCCCC
TLR9	TGAAGACCTCAGGCCCAACTG TGCACGGTCACCAGGTTGT

Supplementary Table 2. Stock concentrations of cytokines used in primary screen

Cytokine	Stock concentration in ng/µL	Cytokines with stock solution of 5ng/µL
IL-13	1	IL-1RA, IL-1β, IL-2, IL-3, IL-4, IL-5,
IL-23	10	IL-6, IL-7, IL-9, IL-10, IL-11, IL12,
IL-29	unknown	IL-15, IL-16, IL-17, IL-18, IL-19, IL-21,
IL-37	unknown	IL-22,IL-25, IL-27, IL-33, TNF-α, TNF-β,
IFN-β	0.2	IFN-α, TGF-β1, MIF, LIF, OSM, TSLP,
IFN-γ	2.5	OPG,CCL1/I-309, CCL2/MCP-1, CCL4
OPN	100	MIP-1β, CCL5/RANTES, CCL7/MCP-3,
CCL3/MIP-1a	2	CCL11/eotaxin, CCL17/TARC

Supplementary Table 2. Stock concentrations of cytokines used in primary screen

Cytokine	Stock concentration in ng/µL	Cytokines with stock solution of 5ng/µL
CCL18/PARC	2	CCL19/MIP-3β, CCL20/MIP-3α, CCL22/
CXCL8/IL-8	2	MDC, CCL27/C-TACK, CXCL4, CXCL5/
Chemerin	50	ENA-78, CXCL9/MIG, CXCL10/IP-10,
Resistin	50	CXCL13/BLC, XCL-1, Adiponectin,
RBP4	500	Adipsin, Leptin, Omentin, G-CSF, M-CFS,
TPO	50	GM-CSF, SCF, HGF, EGF, FGF-Basic, NGF,
SAA-1	50	BDNF, VEGF, sCD14, sCD163, TIMP-1,
sICAM	10	EN-RAGE, Granzyme-B, KIM-1/TIM-1,
sVCAM	10	Cathepsin B, Cathepsin L, Cathepsin
MMP-8	10	S, sPD-1, FAS, FAS-L, IL1-RII, TNF-RI,
MMP-9	50	TNF-RII, sIL-2R, sIL-6R, CRP, Cystatin C,
TREM-1	10	E-Selectin, LIGHT, TL1A, MPO, sSCFR,
IL-18BPA	10	Galectin-9, P-Selectin
IL-1RI	10	
SLP-1	unknown	

Supplementary Table3. Cytokines that did not induce fluid secretion in CFTR-WT organoids

IL-1RA	IL-7	IL-16	IL-25	TNF-RII	MPO	EN-RAGE	TNF-α
IL-1β	IL-9	IL-17	IL-27	sIL-2R	SLPI	TREM-1	TNF-β
IL-2	IL-10	IL-18	IL-33	sIL-6R	sPD-1	Granzyme B	TNF-RI
IL-3	IL-11	IL-19	IL-37	CRP	FAS	KIM-1/TIM-1	IL-1RI
IL-4	IL-12	IL-21	IL-29	Cystatin C	FAS-L	Cathepsin B	IL-1RII
IL-5	IL-13	IL-22	IL-18BPA	E-Selectin	sSCF-R	Cathepsin L	P-Selectin
IL-6	IL-15	IL-23	TL1A	LIGHT	Galectin-9	Cathepsin S	

7

General discussion

Curative treatments for CF patients are still an unmet need. In this thesis, we developed new approaches that can assist the identification and development of CFTR-targeting treatments, and to use those treatments in a more personalized fashion. These included an improved fluorescent sensor for CFTR function measurement in heterologous expression systems. We also applied a primary cell model to study drugs that modulate CFTR function. This primary cell model allowed us to perform small scale screening for existing biological and chemical modulators of CFTR, which may upon pharmacological targeting be useful for treatment of CF. Our data provided important insights into individual mechanisms that may impact the efficacy of such drugs *in vivo*. I here discuss on how the work presented in this thesis can help to achieve the goal of curative treatments for all CF patients, focusing on pharmacotherapies.

Towards cures for all CF patients

For monogenic diseases such as CF, the most important molecular drug target is the mutant gene or its direct products, i.e. the mRNA and protein. Since its original discovery in 1989, almost 2000 *CFTR* mutations have been recognized, indicating that many potential drug targets exist, of which many are highly uncommon. It indicates that multiple treatment approaches need to be developed as to reach all patients.

The primary drug target in CF is CFTR-F508del that is present on at least one allele in ~90% of patients. Recent data indicates that new combinations of at least three drugs are required for optimal repair of F508del [1-2]. However, even in these research settings the overall rescue efficacy remains still insufficient to fully restore function. The limited efficacy of current therapeutics targeting the F508del is also indicated by lack of efficacy in subjects that express only a single F508del allele, and the limited responses in lung function improvement and sweat chloride concentration (SCC) levels in F508del homozygous subjects [1]. It indicates a clear current need for better F508del-restoring drugs, and an optimal drug selection pipeline will help this field moving forward.

For the remaining ~10% of CF subjects with (extremely) rare *CFTR* mutations, it is unlikely that mutation-specific therapy will be directly developed. These subjects may benefit from the extension of drugs being developed for more common CFTR mutations, or from repurposing existing drugs for other indications, or from strategies that restore CF defects in a mutation-independent fashion such as *CFTR* gene therapy or alternative channel modulators.

Towards an optimal CF drug development pipeline

The current small molecules that are in development for CF are selected for repair efficacy of CFTR-F508del, or for a class of mutations (i.e. premature termination codons) that allow treatment of many rare diseases. This field has been driven by the development of high throughput phenotypic cell based assays that enable the cost-efficient screening of large drug libraries for functional repair of mutant CFTR. However, these artificial assay models only partially reflect human biology leading to high false-positive hit rates that need to be filtered using more physiological models.

In CF research the fluorescent-based assays are commonly used to identify novel compounds. Currently, the halide-sensitive YFP is commonly used to monitor CFTR-dependent iodide cell-influx, and has favorable characteristics over previously used fluorescent dyes that label cells with variable efficacy. This assay has been optimized for 384 well format

significantly increasing throughput of the assay [3]. Ratiometric screening methods such as described in chapter 2, allows for the measurement of additional parameters in a single experiment, which can reduce false-positive hit detection. For instance, by confirming identical intensity of the non-halide sensitive mKate protein, one can establish that similar cell amounts remain present after washing and addition of iodide containing buffers. Although false-positive hits are not as detrimental as false-negative results that lead to the exclusion of potential drug candidates, too high false-positive hits result in larger secondary screens that increase the development time and costs. Furthermore, ratiometric measurements also allow different experimental setups, such as end-point measurements that can further increase assay throughput. In applications where cells can move freely in and out of the focal plane, such as in living organisms or cell suspensions, ratiometric measurements are the preferred method of choice (**Chapter 2**, and *e.g.* [4]). Next to the use of this sensor for high throughput screening, we also used this assay readout as an additional tool to indicate CFTR function in epithelial airway-derived cell lines (**Chapter 5**).

Ideally, models that more accurately reflect human biology are integrated in the drug development pipeline as soon as possible. Intestinal organoids that we used throughout this thesis appear optimally suited for drug efficacy testing in comparison with other *in vitro* primary cell models such as upper and lower airway cells or induced pluripotent stem (iPS) cells derived cells. Intestinal organoids are easily obtained from individuals, genetically stable in culture, auto-differentiate, and are relatively easy to maintain in culture. The assay readout uses identical components as the culture medium, is strictly CFTR-dependent, and easy to quantitate. These characteristics allow for long term expansion and multiple repeated testing [5]. Neither primary airway nor iPS cell cultures have all of these traits. Primary airway cells are difficult to maintain in culture and it is even more difficult to culture these cells for extended periods of time, albeit that recent protocols indicate that cells can be expanded up to passage 7 using conditional reprogramming culture conditions [6]. IPS cells may acquire several mutations during the reprogramming or already have these mutations and are not always genetically stable in culture, and the entire procedure to de-differentiate and re-differentiate is complex and lengthy. The afore mentioned characteristics make organoids a very suitable model for drug validation during the preclinical phase of drug development, despite their intestinal origin.

Since pulmonary failure is the main cause of morbidity in CF, primary airway cells have been used to select preclinical drug candidates, as exemplified for the CFTR modulators VX-809 [7] and VX-770 [8]. Any *in vitro* model is not fully recapitulating the *in vivo* conditions, but airway cultures probably reflect the *in vivo* airways better as compared to intestinal organoids. Still, the establishment of *in vitro-in vivo* relationship is not only dependent on the best reflection of the culture. The ability to robustly grow cells, and perform precise CFTR function measurements with limited technical variability will also ensure that sufficient resolution is present to discriminate between different treatment options and patient samples. Organoids swelling correlates well to intestinal current measurements and SCC measurements, and were recently found to correlate with *in vivo* CFTR modulator treatments [9]. Additionally, organoids were used to predict drug responsiveness of a rare, uncharacterized CFTR mutation G1249R which led to successful *in vivo* treatment of patients [5,10-11]. We here demonstrate that PTC124 lacks premature termination codons read-through capacity in organoids (**Chapter 3**), correlating with the lack of *in vivo* efficacy in phase III clinical trials for PTC124 in CF [12] and Duchenne muscular dystrophy [13], where

primary endpoints were not significantly different from placebo. These first steps thus indicate that despite organoids are intestinal of origin, the assay has sufficient accuracy to indicate *in vitro-in vivo* relationships for CF.

In addition to single compound testing (**Chapter 3**), we here demonstrated that organoids can be used to screen small compound libraries such as described in **chapter 5 and 6**. Screening libraries up to 10K compounds should be feasible within one month, using this setup, and it could therefore very well be integrated into hit validation steps and a hit-to-lead program that would consist of various drug selection cycles of several thousands of compounds. It would be technically feasible to even further increase throughput, which could indicate that organoid swelling assay may even be used for primary screening. Slight adaptation of the assay and plate format could further increase the assay throughput up to 50 fold. Utilizing 384 well plates combined with a single time point measurement or endpoint measurement would allow for the identification of the most active hits at an accelerated rate. Furthermore, reducing the sample size by combining multiple compounds in a single well further increase the overall rate of analyzable compounds. These numbers indicate the possibility to perform high-throughput primary screening in organoids. Still, the costs and difficulties of organoid cultures compared to cell line models likely outweigh the advantages of screening in primary cells.

In conclusion, a more favorable approach for CFTR drug development may be designed by performing conventional cell line based high throughput screening to select new hits and integrate organoids at the subsequent hit-to-lead stage. Such a combined approach may more rapidly select the most promising drugs, and reduce time-to-the-clinic for new candidate therapeutics in a more cost-effective manner.

Intestinal organoids to guide drug repurposing and clinical trial design

The arrival of CFTR-repairing drugs announces opportunities for co-treatments that can enhance CFTR activity via alternative mechanisms such as enhancing endogenous CFTR expression levels, or by modulating the channel opening directly through raising cAMP levels. Currently such approaches have not been very actively engaged due to the limited amount of mutations that have consistently been shown to associate with residual function. Many existing drugs may raise CFTR function via the mechanisms as suggested above, indicating that repurposing of these drugs for CF may create quick benefits for subjects.
As described above for CF drug discovery, drug repurposing similarly requires accurate models with sufficient throughput that predict *in vivo* treatment efficacy. **Chapter 5** describes the use of intestinal organoids to identify and treat CF patient subgroups with stimulators of CFTR activity. As we expected, we observed a clear relation between residual function and different CFTR mutations [5]. Organoids harboring CFTR mutations associated with residual function were responsive to β2-agonists. Consistent with known genotype-phenotype relations for the different mutations, we observed a similar efficacy of β2-agonist induced CFTR function in organoids (R117H>A455>F508del>stop). This would indicate that *in vivo* treatment with β2-agonists may activate CFTR in patients expressing CFTR-A455E or -R117H, with higher potential for R117H than A455E.

Upon administrating β2-agonists to patients with residual CFTR function in our open-label phase II clinical trial, only a limited response was observed. This is likely due to the low plasma concentrations as indicated by the limited response of organoids upon exposure to patient plasma after treatment. This plasma assay was created to evaluate plasma

concentration of direct CFTR modulators and can also be used to monitor the concentrations of CFTR activators in plasma (**Chapter 4**). For drug development and clinical trial design, this assay can be used as outcome parameter and is particularly helpful when it is unclear to what extend *in vivo* concentrations can be achieved by treatments. Furthermore, CFTR modulating drug metabolites are also measured in this assay. In our studies, we observed that *in vitro* and *in vivo* responses between β2-agonist treatment in organoids were quite similar, when organoids were stimulated *in vitro* with appropriate concentrations.
The ability to measure CFTR activation by plasma upon oral, but not inhaled, salbutamol intake further corresponded with additional *in vivo* CFTR activity parameters. On this basis, a follow up trial studying the impact of 8 weeks treatment of salbutamol on pulmonary function was initiated using oral dosaging instead of inhalation. The plasma measurements thus helped to design follow-up clinical trials, and we are currently awaiting the results of this trial that aims to demonstrate impact of salbutamol on pulmonary function in subjects with residual CFTR function.

We also performed a small screen to identify inflammatory modulators of CFTR function (**Chapter 6**). Inflammatory modulators have been suggested to regulate mutant CFTR function via diverse mechanisms, but a large scale analysis has currently not been performed. Some important new insights were observed when optimizing the screening assay, including the further enhancement of swelling and TGF-β and p38 inhibitors were removed from the culture medium. From 111 modulators, only type I and type II IFNs were found as CFTR modulators. A previous study with an intestinal cell line already identified type II interferon (IFN-γ), but did not find effects of type I IFN. This further underlines the importance of primary cells in screening efforts. Limiting the impact of IFN *in vivo* on CFTR expression may further help to increase CFTR function, but care must be taken when inflammatory modulators are chronically inhibited by treatment due to potential opportunistic infections. At the same time, exaggerated inflammatory responses are contributing to pulmonary disease severity as suggested from linkage of genetic polymorphisms in inflammatory pathways to pulmonary disease. We therefore suggest that the repurposing of anti-inflammatory mediators may be most effective in patients with CF when the inflammatory inhibitors target both the inflammatory response and ensure that CFTR expression can be maintained.

Future drug repurposing research should focus on identification of medication that either have direct CFTR modulating activity such as the current chemical chaperones VX770 and VX809, or target complementary pathways that enhance CFTR function. Organoids seem to be a suitable model system to investigate these possible novel therapeutics, providing sufficient throughput for screening. Ideally, airway cells can be integrated after initial organoid screening to confirm results in different cell systems. Organoids with particular CFTR mutations can be selected for specific screening purposes. Upon clinical introduction of hits, plasma samples before and during treatment can be used to monitor the *in vivo* delivery of drugs.

Individualizing treatment using organoids

Clinical studies indicate that CFTR-targeting drugs act in a mutation- and patient-dependent fashion. It is clear that the CFTR mutation is a major driver of the response to therapy, and this field will benefit from quick assays that allow the characterization of drug efficacy for unknown mutations. CFTR mutation-independent mechanisms that

contribute to individual variability are largely unknown, but are likely to operate at the pharmacodynamic and pharmacokinetic level.

As discussed above, the organoid assay provides sufficient assay throughput to test all potential CFTR repairing drug combinations for an individual patient. This approach aims to study or predict the tissue response *in vitro*, and therefore studies individual pharmacodynamics parameters and in particular the impact of individual genetic traits. The most appropriate combination based on *in vitro* testing may then be selected for *in vivo* treatment. The most likely strategy is to test the drugs separately and the drugs that show a response are tested for synergistic and additive effects. The most optimal mix of medication can then be selected for each individual patient.

For now, only two drugs are available: Kalydeco (ivacaftor, VX-770 [14]) and Orkambi (ivacaftor and lumacaftor/VX-809 [1]). Now, it is most important to select or deselect patients for these therapies. The current drug ivacaftor was identified based on screens for F508del, and then extended to another class of mutations for which it was found more effective [8]. Currently, cell lines are being used to type CFTR mutations for drugs [15-16]. However, recent data indicate that these cell line models are not always accurate: i.e. subjects harboring one of the mutations selected for inclusion in a ivacaftor extension trial to non-G551D gating mutations did not respond [16-17]. Organoids may be a more accurate model as to demonstrate efficacy of ivacaftor and the combination treatment ivacaftor/lumacaftor in cells of individuals, and appear especially suited for subjects with rare mutations of whom the *CFTR* genotypes are not fully characterized [5].

In addition, it may help to identify high or low responders in groups with identical mutations for who clinical benefit is not directly obvious. Whereas VX-770 has been shown highly effective for particular CFTR mutations, the clinical efficacy of the combination treatment ivacaftor/lumacaftor in patients homozygous for F508del is clearly more limited [1]. Individual parameters generated in organoids may help to deselect subjects from receiving a life-long treatment with potential side-effects for the individual in the absence of clinical benefits and high costs for society [18-20]. Still the validation of such an approach will be difficult; ivacaftor/lumacaftor combination treatment showed the most promising effects on hospitalization and rate of pulmonary exacerbations, but not on shorter acting surrogate endpoints such as FEV_1 and SCC. An extensive validation study as to define the specificity and sensitivity of the *in vitro* measurements is thus required, which needs to focus on relations between surrogate outcome parameters (such as FEV_1 and SCC) and real clinical outcome parameters (such as hospitalization and pulmonary exacerbations).

It still remains unclear how organoids need to be stimulated *in vitro* as to optimally predict the *in vivo* impact of CFTR modulating treatments. In the case of the β2-agonist trial, individual correlations between *in vitro* β2-agonist and *in vivo* β2-agonist treatment are most likely most informative. For CFTR modulators, the use of forskolin to activate CFTR *in vitro* may lead to different outcomes *in vitro* as compared to the activation of CFTR by the endogenous pathways that activate CFTR. Indeed, when β2-agonists and forskolin were directly compared, the response of organoids was clearly different, indicating that patient variability in receptors acting upstream of forskolin modify patient-specific responses of organoids [5]. This points out that additional research is needed to define the most relevant stimuli for organoids to ensure that as much relevant patient variability is incorporated when using this *in vitro* model.

Besides inclusion or exclusion of therapy, organoids can also be used to evaluate

the circulating drug concentrations of an individual after initiation of treatment, and thereby allow to study individual parameters contributing to individual differences in pharmacokinetics (**Chapter 4**). Variation in pharmacokineticts may result from genetic modifiers such as drug metabolizing enzymes in the liver or environmental factors such as additional medication and food intake. Preclinical drug testing combined with the plasma test may help to pinpoint individuals where suboptimal *in vivo* responses are due to poor pharmacokinetic properties, and may lead to easy interventions such as alterations in drug dosage, co-medication or diet as to try to improve the clinical outcome of treatment.

In conclusion, the organoid model may be used to identify drug responsive patients and monitor the *in vivo* drug response. Such a model may shift the current one-size-fits-all treatment strategies based on clinical trials with *CFTR* genotypes selected via cell lines to individual approaches and N-of-1 trials, in which treatments are selected based on individual *in vitro* responses, and adapted *in vivo* based on plasma levels of circulating drugs.

Using organoids to develop therapies for all patients

Currently there is no cure available for every CF patient, mainly due to the large variety of CF-causing gene defects. The approaches used in this thesis and the currently available CFTR-targeting drugs are likely also to be used for other CFTR mutations that are associated with some residual function and (nearly) full-length protein production. However, we need to anticipate that for a limited amount of subjects, the current approaches remain insufficient. Especially subjects with two non-sense mutations, large gene deletions or severe splice mutations remain untreatable by the current approaches.

Currently, a drug candidate for premature termination codons is in development. This drug targets the ribosomal machines, and aims to induce translational readthrough when ribosomes encounter specific stop codon mutations. These drugs, such as PTC124, can in theory be used for every disease caused by nonsense mutations. However, the activity of nonsense suppressing agents was very low in organoids while the activity of PTC124 could not be detected (**Chapter 3**), in agreement with clinical trial data[12,21]. It therefore appears that far more optimal compounds need to be developed, but whether such compounds can specifically lead to suppression of premature stop codons can be questioned.

Gene therapy might be a solution for all CF patients, independent of the mutation. However, efficient delivery of genes to cells remains complicated, and is particularly challenging for CF due to the large size of the entire *CFTR* gene (nearly 200.000 base pairs in length) and the protein coding region (4440 base pair). Recently, a phase IIb clinical trial with aerosolized DNA-lipid complexes was completed that demonstrated stabilization of pulmonary function was the first time that CFTR gene therapy could lead to improved outcomes in CF, but delivery of the CFTR gene into the tissues could not be confirmed. Others are currently developing viral based delivery systems, and organoids have been used to demonstrate that such vector can increase swelling in CF organoids [22]. This area remains therefore promising for treatment of pulmonary complications in all CF patients.

Recently, gene editing has become popular with the arrival of clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated 9 [23-24]. This novel tool allows very precise genome editing and has been used to repair the CFTR-F508del defect in intestinal organoids [25]. The transplantation of *ex vivo* expanded organoids has been successful for several organs such as intestine and liver [26-27]. Although, further optimization of this method could potentially be a cure to restore function to organ-specific

disease expression of CF irrespective of the CFTR mutation, the use of small molecules in the treatment of CF is more likely.

RNA editing molecules that repair specific RNA defects are also under development. These highly sequence specific therapies may be developed for any CFTR mutation. Currently, a RNA editing approach is being developed for the most common mutation, F508del [28], by a Dutch biotech company. RNA editing therapy would require life long treatment, and possible side effects may be limited due to the highly specific action of these drugs. Organoids could also help to confirm the efficacy of these treatments for each individual, but delivery of these RNA-editing molecules to organoids has not been confirmed. This could severely decrease the development time of RNA editing molecules for rare CF-causing mutations.

Approaches that enhance *CFTR* transcription [29-30], modulate splicing [31-32], or modify CFTR conductance [33] remain largely unexplored. Enhancing *CFTR* transcription results in increased amounts of full length CFTR mRNA and enhance CFTR function, which can be relevant in combination with other treatments that have limited efficacy such as the current CFTR modulators, or in subjects with sufficient residual function. Alternate splicing results in decreased amounts of full length mRNA, and influencing the splicing event could result in increased amounts of full length mRNA and subsequent protein expression and function. Efficacy and specificity of this approach remains a challenge. Another method to increase CFTR function of defective channels is to increase or modify the conductance of the channel. VX-770 alters the gating properties of CFTR, but the conductance remains constant [15]. Conductance enhancers, like potentiators, could be used for CFTR mutations that are expressed at the plasma membrane, but have a reduced function. They may also lead to alternative ion transport through the CFTR pore [33], and enhance secretion of bicarbonate that may limit the acidification of epithelial secretions which is crucial for the CF phenotype [34].

Finally, alternative ion channels that bypass CFTR-mediated ion transport could also be a therapeutic targets that can limit the CF phenotype independent of the CF-causing mutation. These include, calcium activated chloride channels (CaCCs), the epithelial sodium channel (ENaC) and solute carriers (SLCs) [35]. CaCC activation by the UTP analog, denufosol, that binds to the purinergic receptor (P2Y2), was unsuccessful in improving pulmonary function in CF patients [36]. This could be due to the relative short pulmonary half-life, and the difficulty of improving pulmonary function in that is above 75%. This finding would suggest that longer acting compounds might still be effective in patients that have worse pulmonary function. Sodium absorption in the absence of CFTR enhances dehydration of the airways, therefore inhibiting ENaC and subsequent prevention of mucus dehydration, could prevent thickening of the mucus layer. However, ameloride, an ENaC inhibitor, failed to demonstrate efficacy in CF-patients [37-38]. The lack of effect can partially be explained by the absence of CFTR activity, which together with ENaC plays an important role in airway hydration [39]. SLC26A9 is also mentioned as potential alternative chloride channel as it expressed in the pulmonary system, and was shown to contribute to constitutive and cAMP depended Cl^- secretion in a CFTR dependent fashion [40-41]. It seems that SLC26A9 mainly enhances functional CFTR, but also has a chloride channel function of it own [42] and with the arrival of CFTR modulating drugs SLC26A9 enhancers could further increase chloride secretion. Targeting alternative ion channels is an ever expanding research field that in the future may benefit CF-patients.

Recommendations for future research

Future research should focus on finding individual combination therapies that maximize CFTR function. Combining multiple correctors [2,43-44] and potentiators [45-46] with distinct modes of action can be used to increase CFTR function levels above those observed for ivacaftor/lumacaftor and ivacaftor. Additional screens using optimal sensors as developed here (**Chapter 2**) may be conducted to identify compounds with distinct CFTR-restoring properties by the addition of existing correctors or potentiators to the initial screening process, or the use of CFTR mutations with specific defects [47].

Organoid drug screens revealed that existing therapies can be identified and repurposed for treatment of CF. The initial studies using β2-agonists (**Chapter 5**) may be expanded, so that all clinically available drugs can be tested for modulation of CFTR function. This may lead to the identification of compound that may directly modulate CFTR function, or indirect through modulation of cellular pathways that impact on CFTR function (**Chapter 6**). Such an approach can lead to quick impact for subjects with specific CFTR mutations.

In addition, the value of intestinal organoids for individualized care need further attention. For subjects with very rare mutations, the organoids can help to select treatments followed by n-of-1 clinical trial designs to demonstrate clinical efficacy. The n-of-1 setting is particularly relevant when objective *in vivo* biomarkers of CFTR function would show only limited responses [48-49]. In subjects with identical CFTR mutations, relations between *in vitro* and *in vivo* parameters must be established, as to show that relative small differences in organoid test results can have meaningful impact on *in vivo* conditions. This may lead to follow up studies when more CFTR modulators enter the field to allow the selection of optimal individual treatments. Additional improvements for individualized drug testing may result from using more physiological stimuli to activate CFTR function as compared to forskolin, and in the end individual airway cultures may provide a better recapitalization of airway disease characteristics.

The plasma test requires further validation in individual treatment settings. Currently, we only used it to study average responses. It remains unclear whether additional plasma factors next to CFTR modulators impact organoid swelling, and what variation is observed when identical subjects with identical treatments are followed up in time using this assay.

Concluding remarks

The recent introduction of CFTR modulators has had a drastic impact on the treatment of CF, but only still for a limited amounts of subjects. The technologies developed and used in this thesis aimed to assist the development of more optimal drugs to modulate CFTR function. The technologies may be implemented at different stages of CFTR drug development. The novel YFP-mKate sensor may generate most impact in large scale drug screening efforts or in specific assays where ratiometric measurements are crucial such as in flow cytometry based assays. Perhaps more importantly, we expand previous studies that developed CFTR function readouts in intestinal organoids [9,50]. We show this model can help to 1) assess drug efficacy at the preclinical phase, 2) screen for potential clinical CFTR modulators, 3) select subgroups of patients for clinical trials, 4) and monitor pharmacokinetic properties of treatments. The studies here further point out that individual organoids are useful at the preclinical and clinical phase of CFTR drug development.

Reference list

1 Wainwright, C. E. *et al.* Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N Engl J Med* **373**, 220-231, (2015).
2 Okiyoneda, T. *et al.* Mechanism-based corrector combination restores DeltaF508-CFTR folding and function. *Nat Chem Biol* **9**, 444-454, (2013).
3 Sui, J. *et al.* Optimization of a Yellow fluorescent protein-based iodide influx high-throughput screening assay for cystic fibrosis transmembrane conductance regulator (CFTR) modulators. *Assay Drug Dev Technol* **8**, 656-668, (2010).
4 Bagnat, M. *et al.* Cse1l is a negative regulator of CFTR-dependent fluid secretion. *Curr Biol* **20**, 1840-1845, (2010).
5 Dekkers, J. F. *et al.* Characterizing responses to CFTR-modulating drugs using rectal organoids derived from subjects with cystic fibrosis. *Sci Transl Med* **8**, 344ra384, (2016).
6 Mou, H. *et al.* Dual SMAD Signaling Inhibition Enables Long-Term Expansion of Diverse Epithelial Basal Cells. *Cell Stem Cell*, (2016).
7 Van Goor, F. *et al.* Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proc Natl Acad Sci U S A* **108**, 18843-18848, (2011).
8 Van Goor, F. *et al.* Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A* **106**, 18825-18830, (2009).
9 Dekkers, J. F. *et al.* A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* **19**, 939-945, (2013).
10 Dijkstra, D. J., Scheffer, H. & Buys, C. H. A novel mutation (G1249R) in exon 20 of the CFTR gene. *Hum Mutat* **4**, 161-162, (1994).
11 Bobadilla, J. L., Macek, M., Jr., Fine, J. P. & Farrell, P. M. Cystic fibrosis: a worldwide analysis of CFTR mutations--correlation with incidence data and application to screening. *Hum Mutat* **19**, 575-606, (2002).
12 Kerem, E. *et al.* Ataluren for the treatment of nonsense-mutation cystic fibrosis: a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Respir Med* **2**, 539-547, (2014).
13 Bushby, K. *et al.* Ataluren treatment of patients with nonsense mutation dystrophinopathy. *Muscle Nerve* **50**, 477-487, (2014).
14 Ramsey, B. W. *et al.* A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *N Engl J Med* **365**, 1663-1672, (2011).
15 Van Goor, F., Yu, H., Burton, B. & Hoffman, B. J. Effect of ivacaftor on CFTR forms with missense mutations associated with defects in protein processing or function. *J Cyst Fibros* **13**, 29-36, (2014).
16 Yu, H. *et al.* Ivacaftor potentiation of multiple CFTR channels with gating mutations. *J Cyst Fibros* **11**, 237-245, (2012).
17 Vertex Pharmaceuticals Incorporated. *U.S. Food and Drug Administration Approves KALYDECO™ (ivacaftor) for Use in Eight Additional Mutations that Cause Cystic Fibrosis*, <http://investors.vrtx.com/releasedetail.cfm?ReleaseID=827435> (2014).
18 Bush, A. & Simmonds, N. J. Hot off the breath: 'I've a cost for'--the 64 million dollar question. *Thorax* **67**, 382-384, (2012).
19 Silverman, E. Orkambi's Slick Unveiling Puts Insurers in a Bind. *Manag Care* **24**, 16-17, (2015).
20 Whiting, P. *et al.* Ivacaftor for the treatment of patients with cystic fibrosis and the G551D mutation: a systematic review and cost-effectiveness analysis. *Health Technol Assess* **18**, 1-106, (2014).
21 Hoffman, E. P. & Connor, E. M. Orphan drug development in muscular dystrophy: update on two large clinical trials of dystrophin rescue therapies. *Discov Med* **16**, 233-239, (2013).
22 Vidovic, D. *et al.* rAAV-CFTRDeltaR Rescues the Cystic Fibrosis Phenotype in Human Intestinal Organoids and Cystic Fibrosis Mice. *Am J Respir Crit Care Med* **193**, 288-298, (2016).
23 Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821, (2012).
24 Ledford, H. CRISPR: gene editing is just the beginning. *Nature* **531**, 156-159, (2016).
25 Schwank, G. *et al.* Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* **13**, 653-658, (2013).
26 Yui, S. *et al.* Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5(+) stem cell. *Nat Med* **18**, 618-623, (2012).
27 Huch, M. *et al.* In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* **494**, 247-250, (2013).

28 Zamecnik, P. C., Raychowdhury, M. K., Tabatadze, D. R. & Cantiello, H. F. Reversal of cystic fibrosis phenotype in a cultured Delta508 cystic fibrosis transmembrane conductance regulator cell line by oligonucleotide insertion. *Proc Natl Acad Sci U S A* **101**, 8150-8155, (2004).

29 Heda, G. D. & Marino, C. R. Surface expression of the cystic fibrosis transmembrane conductance regulator mutant DeltaF508 is markedly upregulated by combination treatment with sodium butyrate and low temperature. *Biochem Biophys Res Commun* **271**, 659-664, (2000).

30 Rowe, S. M. *et al.* Restoration of W1282X CFTR activity by enhanced expression. *Am J Respir Cell Mol Biol* **37**, 347-356, (2007).

31 Nissim-Rafinia, M. *et al.* Restoration of the cystic fibrosis transmembrane conductance regulator function by splicing modulation. *EMBO Rep* **5**, 1071-1077, (2004).

32 Convertini, P. *et al.* Sudemycin E influences alternative splicing and changes chromatin modifications. *Nucleic Acids Res* **42**, 4947-4961, (2014).

33 Jun, I. *et al.* Pore dilatation increases the bicarbonate permeability of CFTR, ANO1 and glycine receptor anion channels. *J Physiol* **594**, 2929-2955, (2016).

34 Shah, V. S. *et al.* Airway acidification initiates host defense abnormalities in cystic fibrosis mice. *Science* **351**, 503-507, (2016).

35 Mall, M. A. & Galietta, L. J. Targeting ion channels in cystic fibrosis. *J Cyst Fibros* **14**, 561-570, (2015).

36 Ratjen, F. *et al.* Long term effects of denufosol tetrasodium in patients with cystic fibrosis. *J Cyst Fibros* **11**, 539-549, (2012).

37 Graham, A. *et al.* No added benefit from nebulized amiloride in patients with cystic fibrosis. *Eur Respir J* **6**, 1243-1248, (1993).

38 Burrows, E. F., Southern, K. W. & Noone, P. G. Sodium channel blockers for cystic fibrosis. *Cochrane Database Syst Rev*, CD005087, (2014).

39 Ghosh, A., Boucher, R. C. & Tarran, R. Airway hydration and COPD. *Cell Mol Life Sci* **72**, 3637-3652, (2015).

40 Avella, M., Loriol, C., Boulukos, K., Borgese, F. & Ehrenfeld, J. SLC26A9 stimulates CFTR expression and function in human bronchial cell lines. *J Cell Physiol* **226**, 212-223, (2011).

41 Bertrand, C. A., Zhang, R., Pilewski, J. M. & Frizzell, R. A. SLC26A9 is a constitutively active, CFTR-regulated anion conductance in human bronchial epithelia. *J Gen Physiol* **133**, 421-438, (2009).

42 Salomon, J. J. *et al.* Generation and functional characterization of epithelial cells with stable expression of SLC26A9 Cl- channels. *Am J Physiol Lung Cell Mol Physiol* **310**, L593-602, (2016).

43 Lin, S. *et al.* Identification of synergistic combinations of F508del cystic fibrosis transmembrane conductance regulator (CFTR) modulators. *Assay Drug Dev Technol* **8**, 669-684, (2010).

44 Boinot, C., Jollivet Souchet, M., Ferru-Clement, R. & Becq, F. Searching for combinations of small-molecule correctors to restore f508del-cystic fibrosis transmembrane conductance regulator function and processing. *J Pharmacol Exp Ther* **350**, 624-634, (2014).

45 Pyle, L. C. *et al.* Regulatory domain phosphorylation to distinguish the mechanistic basis underlying acute CFTR modulators. *Am J Physiol Lung Cell Mol Physiol* **301**, L587-597, (2011).

46 Dekkers, J. F. *et al.* Potentiator synergy in rectal organoids carrying S1251N, G551D, or F508del CFTR mutations. *J Cyst Fibros*, (2016).

47 Phuan, P. W. *et al.* Synergy-based small-molecule screen using a human lung epithelial cell line yields DeltaF508-CFTR correctors that augment VX-809 maximal efficacy. *Mol Pharmacol* **86**, 42-51, (2014).

48 Lillie, E. O. *et al.* The n-of-1 clinical trial: the ultimate strategy for individualizing medicine? *Per Med* **8**, 161-173, (2011).

49 Bollert, F. G. *et al.* Recombinant DNase in cystic fibrosis: a protocol for targeted introduction through n-of-1 trials. Scottish Cystic Fibrosis Group. *Eur Respir J* **13**, 107-113, (1999).

50 Dekkers, J. F., van der Ent, C. K. & Beekman, J. M. Novel opportunities for CFTR-targeting drug development using organoids. *Rare Dis* **1**, e27112, (2013).

8

Nederlandse samenvating

Dankwoord

List of publications

Nederlandse samenvatting

Introductie

Deze thesis gaat over nieuwe diagnostische en therapeutische mogelijkheden bij de behandeling van cystische fibrose (CF) in de volksmond ook taaislijmziekte genoemd. Deze erfelijke aandoening verstoord de werking van veel organen door de aanwezigheid van taai slijm wat vooral in de longen en alvleesklier goed merkbaar is. Door de ophoping van taai slijm in de longen kunnen bacteriën zich er makkelijk handhaven met veelvoudige longontstekingen als gevolg. Bij het bestrijden van deze infecties ontstaat longschade, die langzaam aan het ademen moeilijk maken en uiteindelijk fataal worden voor de patiënten. In de alvleesklier zorgt het dikke slijm voor een ophoping van voedselverteringsenzymen. Doordat de enzymen in de alvleesklier blijven zitten verteren ze deze klier. Dit heeft als gevolg dat voedsel zeer slecht wordt opgenomen door CF-patiënten. Om de gemiddelde levensverwachting van CF-patiënten, ongeveer 40 jaar, te verhogen is een betere medicatie en diagnostiek nodig.

CF wordt veroorzaakt door een mutatie in het CFTR-gen. Een gen is een deel van het erfelijke materiaal (DNA) waarin staat beschreven hoe een eiwit gemaakt moet worden. Voor CF zijn inmiddels meer dan 2000 mutaties beschreven. Als je van zowel je vader als moeder een CFTR-gen met een mutatie krijgt, heb je CF. Dit resulteert in de productie van alleen maar foutief CFTR-eiwit. De productie van het CFTR-eiwit begint in de celkern. Hier wordt een blauwdruk voor de het eiwit gemaakt (mRNA). Na de productie van het eiwit moet het in de juiste vorm worden boetseert of gevouwen. Als het eiwit de juiste vorm heeft mag het naar zijn eindbestemming in dit geval de celwand. Hier functioneert CFTR het als een sluis voor zout moleculen. Bij type mutaties zijn er fouten in het mRNA waardoor er geen (volledig) eiwit wordt gemaakt. Mutaties die voor een zeer afwijkende eindvorm (boetseer fouten) behoren tot de type 2 mutaties. Bij type 3 mutaties gaat de sluis van het CFTR eiwit niet goed open en dicht. Terwijl bij type 4 mutaties de sluis niet de juiste breedte heeft. Bij type 5 en 6 is er wel functionerend eiwit aanwezig in de celwand maar in sterk verminderde mate. De ernst van de ziekte wordt bepaald door het type CFTR mutatie, de andere genen en omgevingsfactoren. “Zware mutaties” van type 1 en 2 resulteren in een ernstig ziekte beeld, terwijl “mildere mutaties” van de andere typen lijden tot een minder ernstig ziekte beeld.

Er zijn nu enkele nieuwe medicijnen die helpen om het CFTR eiwit beter te laten functioneren. Helaas zijn die niet voor alle patiënten effectief, dit is afhankelijk van het type mutatie. Het eerste medicijn dat nu op de markt is, ivacaftor, zorgt dat de sluis van het CFTR eiwit makkelijker open gaat. Het medicijn lumacaftor helpt bij het boetseren van het CFTR eiwit. Ivacaftor en lumacaftor samen lijkt voor een grote groep CF patiënten een positief effect te hebben en zal waarschijnlijk ook snel beschikbaar komen voor patiënten. Een nu nog experimenteel medicijn, ataluren, kan mogelijk bepaalde fouten in de blauwdruk ongedaan maken. Nu is het dus belangrijk om de juiste patiënten met de juiste medicatie te behandelen.

In onze onderzoeksgroep is een nieuwe methode ontwikkeld om de activiteit van het CFTR eiwit te bepalen, met behulp van kleine gekweekte minidarmpjes die groeien uit cellen van een darmbiopt. Minidarmpjes vormen 3 dimensionale ballen van cellen die organoids worden genoemd. Als het CFTR eiwit wordt geactiveerd vult het binnenste van de bal zich met vloeistof. De toename in volume van de organoide is de mate voor CFTR activiteit.

Het onderzoek

In de zoektocht naar nieuwe medicijnen voor CF wordt er gebruik gemaakt van verschillende technieken. De meest voorkomende methode is om met behulp van fluorescentie de activiteit van het CFTR kanaal te meten. In hoofdstuk 2 is een uitbreiding op deze methode beschreven waardoor meer informatie per experiment verzameld kan worden zoals een indicatie van toxiciteit van een geteste stof. Ook is het mogelijk om in bewegende cellen de CFTR activiteit te bepalen. Door deze nieuwe toepassingen kan nieuw medicijn onderzoek worden versneld.

Met de komst van nieuwe medicatie is het belangrijk om te bepalen of deze ook werken voor patiënten met specifieke CFTR mutaties. Een relatief veel voorkomende fout in het mRNA is dat de productie voortijdig wordt afgebroken door een te vroeg stop teken. Stoffen die te vroege stop fouten in de blauwdruk ongedaan kunnen maken, zoals sommige antibiotica en ataluren, zijn getest op organoiden van patiënten met verschillende te vroege stopfouten. De CFTR activiteit nam maar een klein beetje toe bij zeer hoge antibiotica dosering, welke zeer waarschijnlijk te hoog zijn voor therapeutische doeleinden. Als naast de antibiotica ook nog ivacaftor en lumacaftor wordt gegeven zien we een verdere toename in CFTR activiteit. De behandeling met ataluren resulteerde niet in een toename van CFTR activiteit. De algehele CFTR activiteit was zeer beperkt na behandeling met deze stoffen en actievere stoffen moeten dus ontwikkeld worden. Als er in de toekomst effectievere stoffen worden ontwikkeld die te vroege stop fouten ongedaan maken, kan de toevoeging van ivacaftor en lumacaftor een toegevoegd effect hebben.

Met de ontwikkeling van nieuwe stoffen die de CFTR activiteit beïnvloeden is het ook belangrijk om te bestuderen of iedereen wel de juiste dosering krijgt. Met behulp van een bloed monster kunnen we nu in het laboratorium vast stellen of er CFTR reparerende medicijnen in het bloed aanwezig zijn. Door het bloedvocht (plasma) van de bloedcellen te scheiden en het verkregen plasma bij organoiden te doen kan er gekeken worden of er stoffen aanwezig zijn die de CFTR activiteit beïnvloeden. Deze methode is geschikt voor snelwerkende stoffen zoals ivacaftor en voor stoffen die langere tijd aanwezig moeten zijn zoals lumacaftor. Deze test kan in de toekomst gebruikt worden om onder andere interacties tussen medicijnen te bestuderen.

In hoofdstuk 5 hebben we zelf gekeken of we stoffen kunnen vinden die de CFTR activiteit kan verhogen. Hiervoor hebben we verschillende klinisch beschikbare stoffen getest en de groep van de bèta agonisten (astma medicatie) bleek zeer in staat om het CFTR kanaal te activeren. De CFTR activiteit was wel afhankelijk van de aanwezigheid van CFTR eiwit op het plasma membraan. Na bevestiging in andere model systemen zoals, (primair)

gekweekte luchtweg cellen en de methode beschreven in hoofdstuk 2 was er voldoende aanwijzing om deze stof op kleine schaal te testen in patiënten. Hieruit bleek dat bij orale inname voldoende bèta agonist in het bloed aanwezig is om CFTR te activeren dit was onderzocht met de methode beschreven in hoofdstuk 4. Ook bij neuspotentiaal metingen zijn er aanwijzingen dat de CFTR activiteit wordt verhoogd door bèta agonisten. Bij neus potentiaal metingen worden er elektrodes in de neus geplaats en diverse stoffen door de neus geleid die verschillende ion kanalen openen of sluiten, waardoor de stroom die door de cellen heengaat veranderd. Uit die veranderingen kan worden afgeleid of er CFTR activiteit is. Hierdoor is de weg geopend om deze stof op grotere schaal te testen bij CF patiënten die CFTR expressie hebben op de buitenkant van de cel.

CF patiënten hebben veel last van infecties. Bij de bestrijding van infecties door het lichaam komen veel lichaamseigen sloffen vrij die het immuunsysteem beïnvloeden en activeren. In organoiden is onderzocht of deze stoffen de CFTR activiteit beïnvloeden. Voor een belangrijke groep stoffen, de interferonen deze worden uitgescheden na infectie met bacteriën, virussen en schimmels (pathogenen), blijkt dat ze de activiteit van CFTR verminderen. Interferonen zijn cruciaal voor een goede verdediging tegen pathogenen. Daarom is extra voorzichtigheid geboden bij het blokkeren van deze stoffen, omdat er anders geen goede immuunrespons tegen pathogenen komt. Vooral Interferon alfa en bèta lijken goede kandidaten, omdat interferon bèta bij plotseling verergeren van de ziekte voorkomt. Interferon alfa en bèta zijn vooral betrokken bij de bestrijding van virale infecties, terwijl de CF patiënten meer last hebben van bacteriële infecties. Nu moet onderzocht worden of deze stoffen mogelijk een doelwit kunnen zijn voor nieuwe behandelmethode van CF.

Conclusie

Er zijn nog vele nieuwe medicijnen nodig om alle CF patiënten beter te maken. Veel van de nieuwe CF medicatie is mutatie specifiek. Ivacaftor en lumacaftor kunnen samen mogelijk van nut zijn in 60% van de CF patiënten. Voor de andere patiënten zullen dus andere medicijnen gevonden moeten worden. Een deel van deze patiënten kan alsnog gebaat zijn van de ontwikkeling van medicijnen voor de meer voorkomende CF mutaties, uitbreiding van toepassing van bestaande CF medicatie of door de medicijnen die eigenlijk voor andere aandoening gemaakt zijn of niet mutatie specifieke methodes zoals gen therapie of door behandeling via alternatieve ion kanalen.

Door het proces van nieuw medicijn onderzoek efficiënter te maken zal het medicijn ook sneller beschikbaar komen voor patiënten. De methode beschreven in hoofdstuk 2 kan hier een bijdrage aan leveren door bv toxische stoffen te elimineren, wat op termijn scheelt in de hoeveelheid stoffen die nogmaals getest moeten worden. Organoids kunnen ook bijdragen aan een snellere ontwikkeling in medicijnonderzoek. De organoid kweek methode is relatief duur en daarmee minder geschikt voor het onderzoeken van grote collecties van chemische stoffen. Na een eerste screening blijven er kleinere collecties over, hiervoor zijn de organoids wel geschikt, zoals beschreven in hoofdstuk 5/6. Ook om te onderzoeken of individuele patiënten reageren op nieuwe medicijnen of combinaties van medicijnen zijn organoiden erg geschikt, zoals bv beschreven in hoofdstuk 3.

Met de komst van nieuwe medicatie is het belangrijk dat er ook goede diagnostische testen zijn die de biologische activiteit ervan vast te stellen in patiënten. De methode in hoofdstuk 4 is hiervoor ontwikkeld en kan gebruikt worden om veranderingen in medicatie te monitoren. Ook interacties tussen medicijnen, die elkaars effectiviteit beïnvloeden kunnen hiermee worden onderzocht.

Een manier om snel medicijnen beschikbaar te krijgen voor patiënten is door bestaande medicijnen voor de behandeling van andere ziektes te onderzoeken. Astma medicatie kan de activiteit van CFTR verhogen (hoofdstuk 5). Deze methode om meer CFTR activiteit te krijgen is geschikt voor patiënten met CFTR expressie op het plasma membraan dat ook nog functioneel is. Met de komst van ivacaftor en lumacaftor kunnen mogelijk ook de patiënten met de meest voorkomende mutatie hier baat bij hebben.

Een ander behandelmethode die op termijn gebruikt kan worden is gericht op behoud van CFTR functie, door lichaamseigen stoffen te remmen die de activiteit van CFTR verminderen. Deze strategie werkt alleen bij patiënten die CFTR functie hebben, zoals bij de “milde” mutaties, maar ook door gebruik van nieuwe CFTR reparerende stoffen. Door de functie van CFTR te behouden kan mogelijk de achteruitgang van longfunctie worden beperkt. Omdat deze stoffen ook betrokken zijn bij het bestrijden van infecties moeten de voor en nadelen nog wel goed tegen elkaar worden afgewogen.

De hier beschreven diagnostische en therapeutische mogelijkheden kunnen eraan bijdragen dat de levensverwachting van CF patiënten op termijn hoger komt te liggen dan 40 jaar.

Dankwoord

Hier volgt waarschijnlijk het meest gelezen onderdeel van de meeste proefschriften. Dat is eigenlijk heel jammer, daarom adviseer ik: begin voorin met lezen. Daarin heb ik de meeste tijd gestoken en zal daarom stukken beter zijn dan dit dankwoord. Aangezien ik niet goed weet hoe ik alles moet verwoorden heb ik besloten het vooral kort te houden. Ik wil graag iedereen bedanken die heeft bijgedragen aan de totstandkoming van dit proefschrift. Natuurlijk komen voor mij promotor Kors en co-promotor Jeffrey op de eerste plaats, want zonder hun vertrouwen was dit proefschrift er niet gekomen.

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Natuurlijk ben je in Team Beekman nooit alleen, zonder de organisatorische steun van Evelien en woordgrappen van Annelot zou mijn tijd bij Team Beekman heel anders zijn geweest. Mede door jullie harde werken zijn de hoofdstukken mogelijk geworden. Mijn boekje zou er anders hebben uitgezien zonder het harde werken van Florijn, die in ons lab de organoids heeft opgezet. En ik mag natuurlijk de goedlachse Do en droge opmerkingen van Gitte, alles x2, niet vergeten. Samen met jullie heb ik twee hele mooie hoofdstukken gemaakt, waarvoor ik jullie heel dankbaar ben. Ook de “oude” garde van Team Beekman, Marit, Pauline en Jennifer, jullie waren superleuke en gezellige collega’s. Dan zijn er natuurlijk ook nog mijn twee studenten, Saskia en Roel. Saskia na de eerste dag ging het alleen maar beter en jij bent nu maar mooi aan het promoveren. Roel, wat een prestatie! In je stage een artikel bij elkaar pipetteren en publiceren, dat kan niet iedere student zeggen. En natuurlijk de nieuwe leden van Team Beekman, Marne, Gimano en de rest, veel plezier en succes!

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Of course a special thanks to our collaborators in Canada. John Hanrahan and colleagues it was nice working with you and talking to you during the conferences.

Ik heb tijdens mijn AIO periode in meerdere kamers gezeten, wat betekend dat ik vele kamergenoten heb gehad. Bedankt voor jullie gezelligheid, zoals de tafelvoetballen, en gegein met elkaar, Takkie verstoppen. Aangezien dit echt teveel mensen zijn om op te noemen begin ik er maar niet eens aan. Alle leden van het LTI bedankt!!

Tijdens de laatste periode van mijn AIO heb ik een paar mensen iets te weinig aandacht gegeven, met name Edo, Marieke, Timo en Tima. Sorry voor het gebrek aan contact vanaf mijn kant tijdens het schrijven. Maar ik ben heel vereerd dat jullie, Edo en Timo, mijn paranimfen willen zijn. Inge dank je wel voor je steun.

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List of publications

β2-Adrenergic receptor agonists activate CFTR in intestinal organoids and subjects with cystic fibrosis.
Vijftigschild LA*, Berkers G*, Dekkers JF#, Zomer-van Ommen DD#, Matthes E, Kruisselbrink E, Vonk A, Hensen CE, Heida-Michel S, Geerdink M, Janssens HM, van de Graaf EA, Bronsveld I, de Winter-de Groot KM, Majoor CJ, Heijerman HG, de Jonge HR, Hanrahan JW, van der Ent CK, Beekman JM.
Eur Respir J 48, 768-779, (2016)

Limited premature termination codon suppression by read-through agents in cystic fibrosis intestinal organoids.
Zomer-van Ommen DD*, Vijftigschild LA*, Kruisselbrink E, Vonk AM, Dekkers JF, Janssens HM, de Winter-de Groot KM, van der Ent CK, Beekman JM.
J Cyst Fibros 15, 158-162, (2016)

A bioassay using intestinal organoids to measure CFTR modulators in human plasma.
Dekkers R*, Vijftigschild LA*, Vonk AM, Kruisselbrink E, de Winter-de Groot KM, Janssens HM, van der Ent CK, Beekman JM.
J Cyst Fibros 14, 178-181, (2015)

Chronic inflammation and infection associate with a lower exercise training response in cystic fibrosis adolescents.
van de Weert-van Leeuwen PB, Hulzebos HJ, Werkman MS, Michel S, Vijftigschild LA, van Meegen MA, van der Ent CK, Beekman JM, Arets HG.
Respir Med 108, 445-452, (2014)

Cell-penetrating bisubstrate-based protein kinase C inhibitors.
van Wandelen LT, van Ameijde J, Ismail-Ali AF, van Ufford HC, Vijftigschild LA, Beekman JM, Martin NI, Ruijtenbeek R, Liskamp RM.
ACS Chem Biol 8, 1479-1487, (2013)

A novel fluorescent sensor for measurement of CFTR function by flow cytometry.
Vijftigschild LA, van der Ent CK, Beekman JM.
Cytometry A 83, 576-584, (2013)

CFTR-mutation specific applications of CFTR-directed monoclonal antibodies.
van Meegen MA, Terheggen SW, Koymans KJ, Vijftigschild LA, Dekkers JF, van der Ent CK, Beekman JM.
J Cyst Fibros 12, 487-496, (2013)

* or # authors contributed equally

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