Precision medicine for Cystic Fibrosis using intestinal organoids

Peter van Mourik

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Precision medicine for Cystic Fibrosis using intestinal organoids

Precisiegeneeskunde voor Cystic Fibrosis door middel van intestinale organoïden
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
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in het openbaar te verdedigen op
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door

Peter van Mourik

geboren op 6 maart 1990 te IJsselstein

Promotoren:

Prof. dr. C.K. van der Ent
Prof. dr. J.M. Beekman

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General Introduction

Adapted from:
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GENERAL INTRODUCTION

Recent advances in adult stem cell biology have resulted in the development of organoid culture technologies using a variety of tissue sources such as intestine, lung, and kidney [1]. Organoids are three-dimensional, multi-cellular structures that recapitulate tissue features of the parental organ and are usually grown from donor tissue fragments [1]. As organoids are functional expressions of individual genomes, these cultures are particularly useful to understand how genetic factors contribute to individual disease. As such, they are used to study hereditary diseases like cystic fibrosis (CF), and more common diseases such as cancer where genetics can influence disease severity and drug efficacy [2,3].

Intestinal organoids have been on the forefront of these developments as culture methodology was first developed for this tissue source [4]. For CF, human intestinal organoids can be grown from intestinal crypt fragments that are isolated from rectal biopsies. Taking rectal biopsies is typically an innocuous procedure that can be performed in all age groups (including newborns) without a need for anesthesia [5,6]. Intestinal organoids can be generated with high individual success rates, which facilitates access to tissue that can be expanded, stored in liquid nitrogen and can be thawed and continuously cultured for more than 6 months while preserving individual functional phenotype [7]. While many labs worldwide have adopted organoid culturing [8], few laboratories have expertise in using intestinal organoids to study CF. Here we focus on applications of intestinal organoids for CF and emphasize important future directions that can help to develop effective therapies for all people with CF.

HOW DOES IT WORK?

Genetic variability of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene can result in misfolding and malfunction of the CFTR protein. This subsequently dysregulates epithelial ion and fluid transport, resulting in CF disease manifestations. CFTR plays a dominant role in rapid fluid secretory responses in the large intestine that are evoked by cyclic AMP (cAMP) inducing stimuli and its activation can lead to secretory diarrhea. Chloride is the main driver of this rapid fluid secretory response [9,10].

CFTR is expressed on the apical membrane that lines the internal lumen of rectal organoids. CFTR activation by cAMP-raising agents such as forskolin leads to chloride transport into the organoid lumen that is accompanied by luminal water secretion through osmosis (Figure 1). Two functional assays have been developed

to quantify CFTR function in intestinal organoids that rely on the luminal chloride secretion and coupled water transport.

First, incubation of organoids with forskolin leads to rapid luminal fluid secretion through CFTR activation that causes whole organoid swelling within 60 minutes. The forskolin-induced swelling (FIS) phenotype is absent in human and mouse organoids lacking functional CFTR gene products (e.g. two class I mutations or *Cftr* knock out), and is inhibited by chemical CFTR inhibitors, supporting full CFTR-dependency of the FIS readout [10]. The resulting swelling of organoids is quantified by live confocal microscopy after organoid labelling with calcein-green. The relative size increase of all organoids in a well is calculated over time using 10 minute intervals. A relative swelling curve is generated from these data and an area-under-the-curve of this relative swelling is calculated to compare conditions (e.g. different drugs, different donors) in a single graph [11].

Second, steady-state differences in luminal phenotype exist between healthy control and CF organoids independent of forskolin. Healthy control rectal organoids have large fluid-filled lumens, suggesting the presence of functional CFTR and physiological cAMP signalling during standard culture conditions leading to luminal salt and fluid transport [12]. CF organoids do not have lumens that are easily recognized upon visual inspection. This phenotypical difference can be quantified before a FIS assay is performed by manually drawing in the lumen of calcein-labeled organoids in the baseline images, and subsequently expressing the lumen area as percentage of total area. Whereas CF organoids have steady-state lumen areas (SLA) between 0-10 percent of total organoid area, healthy control SLA is between 40-80%. While SLA is mostly CFTR-driven, some variability in this phenotype exists at the 0-10% range independent of CFTR genotype.

FIS and SLA are complementary assays. FIS has considerable throughput and quantifies CFTR function at levels associated with CF disease and treatment thereof with current CFTR modulators [7,10]. As FIS measures relative size increase, it requires that the initial starting sizes are comparable. This is true for CF organoids, but at larger SLA (~>25%), relative size increases are underestimated when 2D area measurements are performed [7]. FIS of CF and healthy controls are therefore not directly comparable. SLA facilitates comparison between CF and healthy control organoids, but has limited resolution to discriminate at lower CFTR function levels associated with severe CF disease [7].

Both FIS and SLA have only recently been developed, and technological expertise is currently available in a select number of laboratories. The CFTR dependency of FIS has been confirmed by independent other CF (e.g. De Boeck et al., Amaral et al.) and non-CF laboratories in intestinal [13] and non-intestinal tissues [14]. To further standardize multicenter research, biopsies can also be shipped to a centralized laboratory for processing, which is feasible within 48-72 hours after obtaining the biopsy. Growing organoid structures for FIS experiments takes approximately four weeks, and at that point organoids can be stored in liquid nitrogen for future use. When in culture, FIS and SLA assays can be performed weekly, and repeated measurements over several weeks can be performed to ensure reliability of measurements.

WHAT IS THE CURRENT USE OF ORGANOIDS IN STUDYING CYSTIC FIBROSIS?

Intestinal organoids are used to better understand how CFTR function or manipulation thereof by therapeutic interventions can impact on individual clinical phenotype (Figure 1). Additionally, the recently funded HIT-CF project (http://www.hitcf.org) will use organoids to design clinical trials by pre-treatment stratification of in vitro responders.

Clinical studies indicate that organoid FIS is relevant to characterize CFTR function of rare CFTR variants. Many of the currently known 2000 CFTR variants have not been characterized, which complicates the prediction of individual disease phenotype. Moreover, limited data regarding drug effects on rare genotypes prevents access to existing and new CFTR-mutation specific treatments. Pilot studies indicate that organoids could help both as individual biomarker of CFTR function to predict individual disease phenotype [15], and could help characterize rare mutations for treatment response [7].

Organoids and clinical disease phenotype

Organoid FIS reflects residual CFTR function and correlates with predicted phenotypic characteristics of the CF genotype [7,15], and other biomarkers of CFTR function such as Sweat Chloride Concentration (SCC) [7,15] and intestinal current measurements (ICM) [15,16]. While Nasal Potential Difference (NPD) is another important clinical biomarker of CFTR function, no data on the correlation between organoid FIS and Nasal Potential Difference is currently available.

The first study to directly compare organoid FIS and clinical phenotype prospectively included 34 newborns with CF. Newborns were clustered into low FIS or high FIS. Low

FIS in organoids was related to increased pulmonary and pancreatic disease parameters at the age of 1 year [15]. Interestingly, in cases where SCC and ICM disagreed, FIS appeared to correctly align with the clinical indicators [15]. This study suggests that the full CFTR dependency of FIS and the 48 datapoints we typically gather (by titrating forskolin and repeating measurements) to type individual CFTR residual function may provide a higher accuracy and precision as compared to other biomarkers of CFTR function. The precise measurement of CFTR function also suggests that a further refinement of residual function classification is possible. Since these early studies are small, it is of yet unclear how well the model predicts long-term clinical risk. Follow-up studies in larger cohorts are needed to validate the use of organoids for predicting disease phenotype in individuals with rare mutations, and predicting phenotypic variability between individuals with identical CF-causing mutations.

Organoids for studying drug efficacy

Preclinically, organoids are being used to identify and develop CFTR modulating drugs and explore mechanisms associated with differences in CFTR function. Pharmaceutical companies use intestinal organoids in their drug development pipeline [17,18]. During initial high-throughput-screening, many different chemical structures are discovered that could work as CFTR-modulating drugs. Organoids can be a useful tool to efficiently validate lead compounds, because of their medium-high throughput, sensitivity to drug effects in combination with the fact that they express endogenous CFTR. Organoids are used to test the potency of single drugs, but also to compare the efficacy of different combination treatments [17,18].

Moreover, studies by Dekkers et al. in organoids indicated that both the CFTR mutations and additional patient-specific genetic differences modify response to CFTR modulators [7,10,19]. By studying the effects of a range of drugs on different genotypes, the optimal CFTR modulating drug and their potency for each genotype can be identified for further clinical studies [19,20].

Several studies have highlighted the translational potential of organoids. Genotype-specific effects of ivacaftor and lumacaftor/ivacaftor in organoids correlate with clinical trial data at group level [7]. The failure of a phase III clinical trial with Ataluren in subjects with stop-codon mutations was preceded by a report on the absence of drug activity in the organoid model [21,22]. These group-based observations indicate that organoids can help to characterize the response of mutations to particular treatments. Such an approach would complement current CFTR ‘theratyping’ efforts that mostly rely on cell line expression systems in which CFTR mutations are introduced. Based on quantitative and qualitative differences in therapy response between organoid

subgroups (e.g. comparing average p.Ala455Glu/p.Phe508del, p.Asn1303Lys/p.F508del or class I/p.Phe508del responses), mutation-specific phenotypes with different therapeutic vulnerabilities can be deduced [19]. These theratyping efforts have no direct relation to the individual patient, but rely on the coupling of individual CFTR genotype to the cell-based data.

Recent proof-of-concept in two patients with ultra-rare mutations showed clear in vitro-in vivo correlation in response to treatment with ivacaftor [7]. This study indicates that organoids could provide a functional readout, which might even integrate both the patient-specific CFTR mutations and additional genetic variation that modifies the response to treatment. If these results are validated in subsequent studies, this would suggest that a direct test on the patient organoid can be used to characterize the response to treatment, independent of any a priori knowledge on the CFTR genotype.

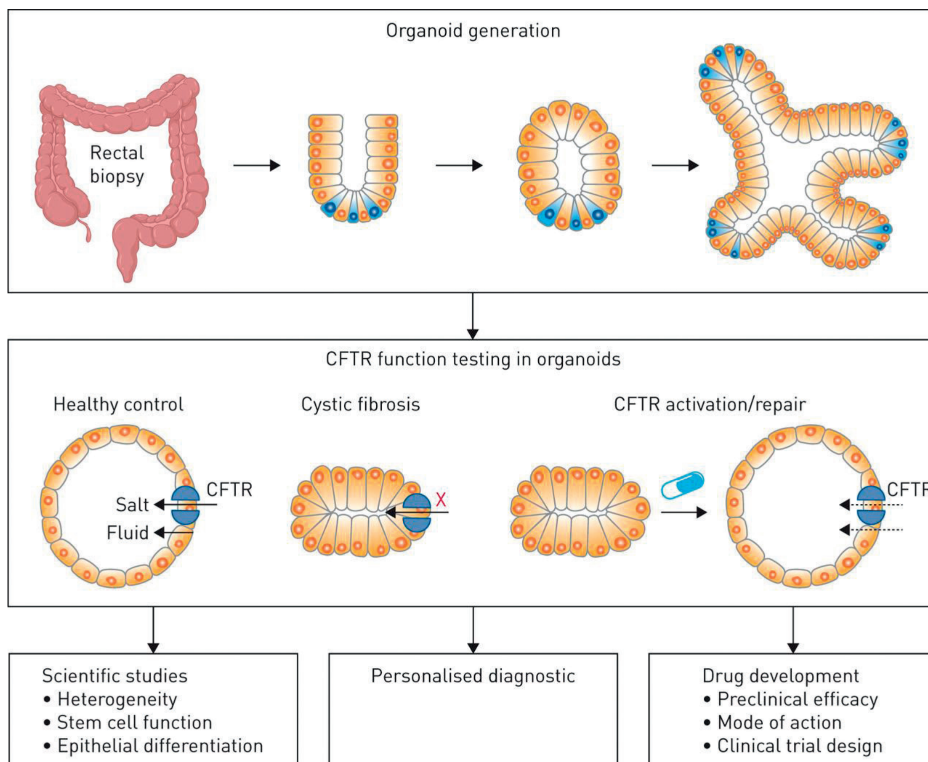

Figure 1. Potential applications of intestinal organoids to study cystic fibrosis. Intestinal organoids can be generated from rectal biopsies. In organoids, robust cystic fibrosis transmembrane conductance regulator (CFTR) function measurements can be performed based on phenotypic differences between organoids and the observation that repair and activation of CFTR causes swelling of organoids. This model can be used in preclinical and clinical studies with the potential to obtain patient-specific information on disease severity and CFTR-modulator drug response.

FURTHER DEVELOPMENT OF ORGANOIDS AS A TOOL IN CYSTIC FIBROSIS RESEARCH

Since the use of intestinal organoids in Cystic Fibrosis is quite recent, standardized protocols have not been adopted and validity and reproducibility of the FIS assay in organoids has not been assessed. Studies exploring these aspects could further elucidate the qualities of organoids as biomarker in Cystic Fibrosis.

Current studies with organoids support that additional classification of residual function may be possible. CFTR function is likely a biological continuum that is mostly dependent on CFTR genotype and further modified by individual genetic and environmental factors. The first clinical validation studies showed how relatively large differences in residual function (albeit all in the CF domain) correspond with clinical manifestations [15]. Whether more subtle differences in organoid swelling (e.g. between organoids from people with identical CF-causing mutations; or in conditions of borderline CF and CFTR-related disease) also associate with clinical phenotype and drug response should be investigated in clinical correlation studies.

AIMS OF THIS THESIS

The aim of this thesis is to assess intestinal organoids as a tool for CFTR-modulator development and precision medicine. More specifically, the following research questions are addressed:

I. Can intestinal organoids improve our knowledge on the interaction between CFTR-modulators and CFTR-genotypes?
II. Could intestinal organoids be internationally implemented for CF research and clinical care?
III. How does CFTR-modulator efficacy in intestinal organoids relate to clinical response in individual patients?

In part 1 of this thesis, we focus on the use of intestinal organoids to further characterize the interaction between CFTR-genotype and CFTR-modulators. **Chapter 2** explores whether variability in CFTR-mRNA expression between donors might influence CFTR function and ivacaftor efficacy in organoids with the R117H-CFTR genotype, while **Chapter 3** examines the responsiveness of several CFTR-genotypes to combinations of potentiators.

Next, part 2 is focused on improving the implementation of intestinal organoids. Therefore, **chapter 4** contains a protocol that has been implemented in four laboratories, and can be used worldwide to reduce inter-lab variability in both execution and results of the FIS assay, while **chapter 5** examines the safety and success rate of rectal biopsy procedures for establishing intestinal organoid cultures.

Part 3 explores the use of the FIS assay in intestinal organoids as a biomarker for clinical CFTR-modulator response by correlating FIS assay results with clinical trial data. **Chapter 6** is an explorative study in patients homozygous for the F508del mutation, where we examine the correlations between clinical effects of lumacaftor/ivacaftor treatment on several endpoints and intestinal organoid FIS when treated with lumacaftor/ivacaftor. **In chapter 7** we assess whether intestinal organoid FIS response to curcumin, genistein, ivacaftor and lumacaftor/ivacaftor correlates with clinical response to these drugs in patients with a range of CFTR genotypes. Next, **chapter 8** contains the study protocol of the HIT-CF Organoid Study, the first part of the HIT-CF Europe project. HIT-CF Europe aims to provide access to CFTR-modulators for patients with rare CFTR-mutations using organoids.

Chapter 9 is a discussion of the results of the previous chapters and their combined implications for the use of intestinal organoids in the field of Cystic Fibrosis.

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R117H-CFTR function and response to VX-770 correlate with mRNA and protein expression in intestinal organoids

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ABSTRACT

Introduction: Variability in disease severity and CFTR modulator responses exists between patients with identical CFTR genotypes. Here, we characterized transcription, translation and function of R117H-CFTR using intestinal organoids and correlated them with in vitro responses to ivacaftor (VX-770).

Methods: Organoids were generated from individuals possessing at least one R117H-CFTR allele. The forskolin-induced swelling (FIS) assay was used to measure CFTR function and response to VX-770 treatment. R117H-CFTR protein and mRNA expression levels were determined in parallel and Pearson's correlation coefficients were assessed.

Results: Variability in R117H-CFTR FIS responses was observed and correlated significantly with mRNA and protein expression. Response to VX-770 treatment in organoids correlated with mRNA and protein expression as well.

Discussion: Our results indicate that gene expression, protein expression and CFTR function are strongly correlated in organoids from people with CFTR-R117H-7T/9T, which may suggest that CFTR gene expression may have consequences for CF diagnosis, prognosis and therapeutic benefit.

INTRODUCTION

Cystic fibrosis (CF) patients with identical *cystic fibrosis transmembrane conductance regulator (CFTR)* gene mutations can display heterogeneity in disease severity, which is particularly evident in patients harbouring the R117H-CFTR (p.Arg117His, c.350G>A) mutation. Disease severity in these individuals ranges from being asymptomatic to severe lung function decline comparable to CF patients with homozygous F508del-CFTR (p.Phe508del, c.1521_1523delCTT) mutations [1,2].

A genetic influence contributing to disease heterogeneity is the length of a poly-thymidine (poly-T) tract (5T, 7T or 9T) present in cis with R117H-CFTR [1,3], which affects splicing efficiency and mRNA transcript availability [4,5]. A 5T tract is considered to be a contributor to disease severity, whereas 7T has been associated with 'milder' CF. In this latter (sub)group widely differing symptoms have been reported [3,6] resulting in problems related to patient counselling and treatment strategies.

Currently, the R117H-CFTR mutation is one of 33 mutations for which the CFTR potentiator ivacaftor (VX-770) has been approved [7]. Its efficacy, however, differs between individuals possessing similar mutations [8], which indicates the need for individual drug response prediction. To gain insight into mechanisms contributing to variability in response to CFTR modulator treatment we characterized (i) transcription, (ii) translation and (iii) function of R117H-CFTR using patient-specific intestinal organoid cultures and correlated these datasets with CFTR function restoration upon VX-770 treatment.

MATERIAL AND METHODS

Colon biopsies were obtained for diagnostic purposes as part of CF care, or study participation with approval of the Ethics Committees of the University Medical Center Utrecht and the Erasmus Medical Centre Rotterdam. Organoid generation and culturing was performed as described previously [9–11].

Paired samples of seven or eight days old organoid cultures from multiple passages (8-12 wells of a 24-wells cluster) were generated of which (i) 30% was used for RNA isolation, (ii) 30% for protein extraction and (iii) 40% for organoid seeding, the forskolin-induced swelling (FIS) assay and continuation of the organoid cultures. RNA/protein isolation, gene expression studies, Western Blot analysis and the FIS assay are described in the Supplementary Methods. Experiments were repeated at three different culturing time points.

Statistical analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software, La Jolla California USA). Correlations were determined by Pearson correlation coefficient (Pearson's r) and linear regression analysis. P-values were reported based on two-sided tests.

RESULTS

Fourteen R117H-CFTR (13 with known 7T-9T poly-T tracts and one with unknown poly-T status, of which nine compound heterozygous for F508del-CFTR), one class I (c.1679+1G>C/c.1679+1G>C), one class III (p.Phe508del/p.Ser1251Asn) and one wild type CFTR organoid culture were included in this study (Supplementary table 1).

First, we assessed (residual) CFTR function using the FIS assay. Upon 0.05 µM and 0.128 µM forskolin treatment variability in FIS was observed between R117H-CFTR organoids cultures (AUC range: -88 – 586 AUC and 193 – 1452 AUC, respectively, Figure 1A and 1B). VX-770 treatment of the R117H-CFTR organoids resulted in increased swelling of all organoids compared to their non-treated counterparts with a large variability in VX-770 response (AUC range: 252 – 1922 and 602-2524 at 0.05 µM and 0.128 µM forskolin treatment, respectively, Figure 1A and 1C).

Next, we determined whether differences in residual CFTR function and response to VX-770 correlated with CFTR protein expression by measuring glycosylated CFTR (C-band) expression. CFTR expression was corrected for sample loading using endogenous heat shock protein 90 (HSP90) and normalized to wild-type CFTR protein expression. As can be seen in Figures 2A and 2B, C-band protein expression was highly variable between R117H-CFTR organoid cultures (relative expression range: 0.23 – 2.00).

We performed gene expression studies to quantify the allele-specific contribution of R117H-CFTR to the observed variability in CFTR function and response-to-therapy. R117H-CFTR mRNA expression results were normalized using *ACTB* and *YWHAZ* housekeeping genes and converted into relative quantities using the R117H-homozygous organoid culture as wild type organoids do not possess the R117H-CFTR allele. As expected, R117H-CFTR mRNA expression was highest in the R117H-homozygous organoid culture which possesses two alleles that contribute to the mRNA expression (Figure 2C). Clear variability in mRNA expression between organoid cultures with a single R117H-CFTR allele (relative expression range: 0.18 – 0.49) was detected. R117H-CFTR mRNA expression and CFTR C-band protein expression were strongly correlated ($r=0.92$, $p<0.0001$, Figure 2D). To investigate

the contribution of the R117H-homozygous organoid measurements to the observed correlation the analysis was repeated without including this data and still revealed a strong correlation (r=0.88, p<0.0001, Supplementary figure 1A).

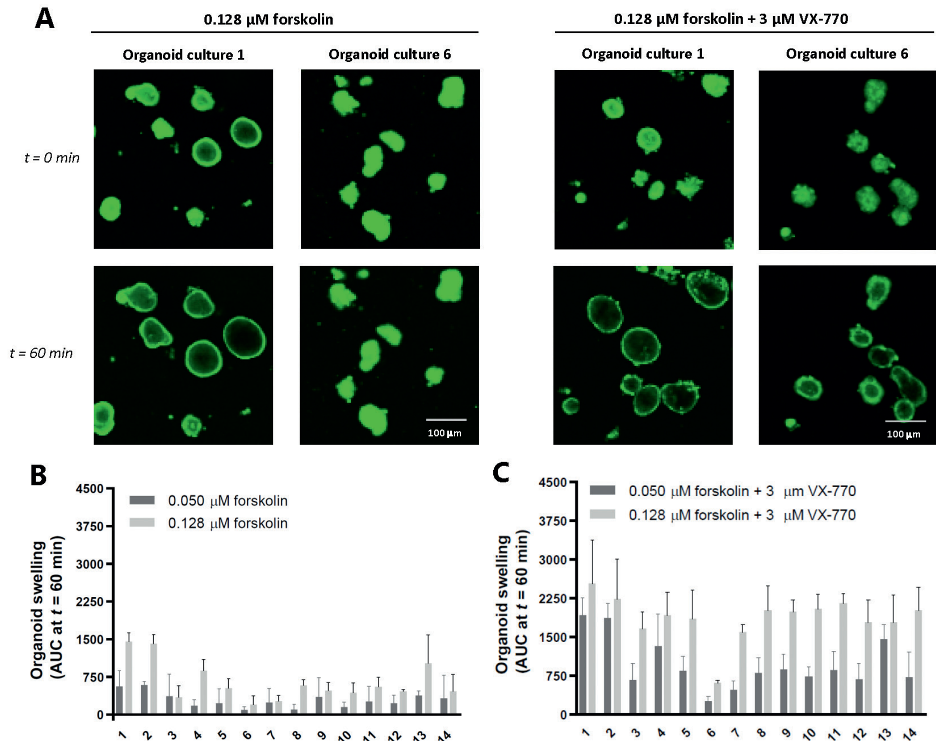

Figure 1. Forskolin-induced swelling (FIS) of R117H-CFTR organoids. (A) Representative confocal images of calcein green-labelled and forskolin-induced swelling of organoids harbouring the R117H-CFTR mutation demonstrating differences in FIS and treatment responses. Organoids of culture 1 have a clear lumen at t = 0 min, while organoids of culture 6 do not have a visible lumen at t = 0 min. Upon VX-770 treatment FIS occurs in both cultures. (B) AUC quantification of 0.05 µM and 0.128 µM forskolin-induced organoid swelling after 60 min. (C) AUC quantification of 0.05 µM and 0.128 µM forskolin-induced organoid swelling after 60 min of organoids treated with 3 µM VX-770.

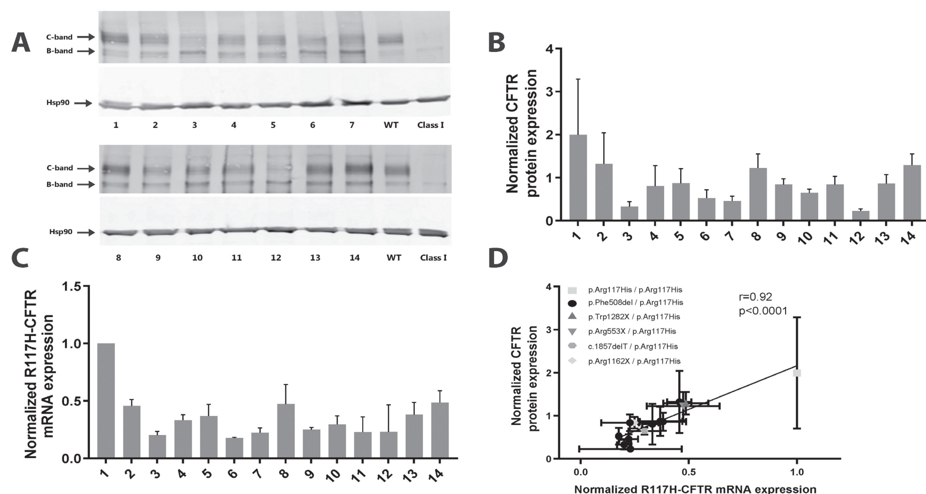

Figure 2. Protein and mRNA expression in R117H-CFTR organoids. (A) Representative images of the western blot analysis demonstrating CFTR C-band, CFTR B-band and HSP90 loading control protein expression. (B) CFTR protein (C-band) expression corrected for sample loading using HSP90 protein expression and normalized to protein expression in the wild type (WT) sample. (C) R117H-CFTR mRNA expression normalized for ACTB and YWHAZ gene expression and converted into relative quantities by normalization to organoid 1 (R117H-CFTR/R117H-CFTR). (D) Correlation analysis of normalized R117H-CFTR mRNA expression with normalized CFTR protein expression.

Finally, we investigated the relations between FIS, mRNA and protein expression in R117H-CFTR organoids. Correlation was especially evident between 0.128 μM FIS and R117H-CFTR mRNA and protein expression (r=0.75, p<0.01 and r=0.74, p<0.01, respectively, Figures 3A and 3B). This correlation persisted with the 0.05 =.01, respectively, Figures 3A and 3B). This correlation persisted with the 0.05 μm FIS results and mRNA and protein expression, albeit less strongly (r=0.57, p=0.04, and r=0.56, p=0.04, Supplementary figure 1B and 1C, respectively). VX-770-treated FIS at 0.05 μM forskolin demonstrated a correlation with mRNA (r=0.72, p<0.01, Figure 3C) and protein (r=0.72, p<0.01, Supplementary figure 1D) expression, which was also present after correcting for vehicle-treated organoid swelling (r=0.69, p<0.01, Figure 3D and r=0.69, p<0.01, Supplementary figure 1G, respectively). FIS of VX-770-treated organoids with 0.128 μM forskolin did correlate with mRNA and protein expression (r=0.57, p=0.03, and r=0.6, p=0.02, Supplementary figure 1E and 1F, respectively) but after correcting for vehicle-treated organoid swelling these correlations were lost (see Supplementary figure 1H and 1I).

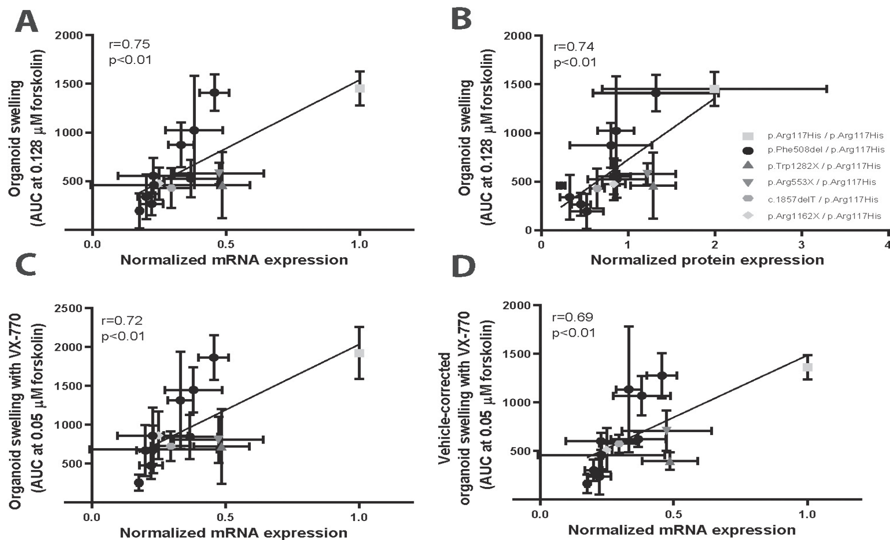

Figure 3. Correlation analyses between FIS, mRNA and protein expression in R117H-CFTR organoids. Correlation analyses was performed for (A) normalized R117H-CFTR mRNA expression and organoid swelling after stimulation with 0.128 μM forskolin, (B) normalized CFTR protein expression and organoid swelling after stimulation with 0.128 μM forskolin, (C) normalized R117H-CFTR mRNA expression and organoid swelling in the presence of 3 μM VX-770 and 0.05 μM forskolin stimulation, and (D) normalized CFTR mRNA expression and vehicle-corrected organoid swelling in the presence of 3 μM VX-770 and 0.05 μM forskolin stimulation.

DISCUSSION

Recent studies using airway epithelial cells and intestinal organoids found strong correlations between residual CFTR function and response to CFTR modulators across different genotypes [10,12]. Our current study adds to these findings that within one genotype, i.e. one R117H-CFTR allele, the CFTR modulator response (VX-770) seems to be primarily influenced by differences in mRNA expression. These observations warrant exploration of the relationship between CFTR mRNA expression and CFTR modulator responsiveness within individual genotypes in more detail.

We observed variability in FIS responses, which cannot be attributed to in vitro exposure differences as all organoid cultures were cultured simultaneously under identical conditions and processed in parallel. Clinical heterogeneity between patients harbouring R117H-7T has been reported [13,14] but in vitro studies into possible non-environmental contributors are lacking. The strong correlation between mRNA expression and CFTR function in our study suggests that (epi)genetic factors

influencing mRNA expression could impact R117H-CFTR function. Whether mRNA expression variability in the intestinal epithelium reflects variability in other tissues, e.g. airway epithelium, will require further investigation as mRNA expression differs between tissues [4]. We do not know if the observed large variability in mRNA expression in our studies might affect the utility of mRNA expression as predictive tool for CFTR function but our previous studies indicated that differences in organoid CFTR function translate well to the in vivo situation [10,15,16].

None of the R117H-CFTR subjects of which organoid responses were investigated in this study were treated with ivacaftor. As such, we could not correlate clinical disease and ivacaftor responsiveness. Exploratory correlation analyses of R117H-CFTR mRNA expression and 0.128 μm FIS with sweat chloride concentration (SCC; n=12), showed a trend towards correlation (r=-0.44, p=0.16, r=-0.40, p=0.17, supplementary figure 1J and 1K, respectively), which was absent for 0.05 μm FIS (Supplementary figure 1L). CFTR protein expression was significantly correlated to SCC (r=-0.6, p=0.03, supplementary figure 1M). Additional studies are required to determine the clinical and diagnostic utility of using CFTR mRNA expression as a potential predictor of therapy effectiveness.

Interestingly, the correlation between mRNA expression and VX-770 FIS responses using 0.128 μM forskolin was absent (data not shown). A recent study by Cui *et al.* [17] demonstrated that high phosphorylation levels of CFTR can obscure potentiator responses, which could explain the absence of this correlation in our study due to this ceiling effect.

A caveat in this study is that we have used available genetic information and did not perform additional testing to confirm the poly-T status or determine the TG repeats in the organoid cultures. It is known that the poly-T tract variant influences CFTR splicing [4,5,18]. As assays to determine on which allele the 7T tract is located are not readily available we assumed that 7T was in cis with R117H based on available literature [19,20]. Studies have demonstrated that the 5T variant results in increased exon 10 skipping, i.e. less full-length CFTR transcript, and a more severe disease phenotype than the 7T variant [1,3,21]. Our current study, however, demonstrated variable mRNA expression of R117H/7T-CFTR in subjects with this genotype as well suggesting that the amount of R117H-CFTR mRNA might be predictive of CFTR function in vivo.

Our experiments were only performed in organoids expressing R117H-CFTR. It is unclear whether these results can be extrapolated to other genotypes (or R117H in cis

with 5T, 9T) and CFTR modulator treatments. Since transcript availability determines the quantity of CFTR that can be restored, variability in mRNA expression might contribute to the heterogeneity in phenotype and treatment responses observed with other CFTR genotypes. This could also indicate that interventions that increase CFTR gene expression may have therapeutic benefit.

Linking *CFTR* mRNA expression to clinical disease severity and variability in drug response is needed to determine the clinical implications of our findings. This study would suggest that differences in mRNA expression may be useful as an additional diagnostic tool for both disease prognosis and (individual) response to CFTR modulator treatment(s).

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SUPPLEMENTARY DATA

Supplementary table 1. Genotypes of intestinal organoids used in this study.

Organoid number	Mutation 1	Mutation 2	Poly-T-tract
1	p.Arg117His	p.Arg117His	7T
2	p.Arg117His	p.Phe508del	7T-9T
3	p.Arg117His	p.Phe508del	7T-9T
4	p.Arg117His	p.Phe508del	Unknown
5	p.Arg117His	p.Phe508del	7T-9T
6	p.Arg117His	p.Phe508del	7T-9T
7	p.Arg117His	p.Phe508del	7T-9T
8	p.Arg117His	p.Arg553X	7T-9T
9	p.Arg117His	p.Arg1162X	7T-9T
10	p.Arg117His	c.1857delT	7T-9T
11	p.Arg117His	p.Phe508del	7T-9T
12	p.Arg117His	p.Phe508del	7T-9T
13	p.Arg117His	p.Phe508del	7T-9T
14	p.Arg117His	p.Trp1282X	7T-9T

Forskolin-induced swelling (FIS) assay

FIS was performed as described previously [1–3]. In short, organoids were seeded in a 96-well tissue culture-treated plate in 4 µl 50% Matrigel® droplets immersed in 50 µL culture medium. Following overnight incubation at 37°C/5% CO_2 organoid cultures were incubated with 750 nM calcein green (Life Technologies: Gibco) for 30 minutes at 37°C/5% CO_2. Forskolin (0.050 µM or 0.128 µM) (Sigma), together with 3 µM VX-770 (Selleck Chemicals LLC) or carrier (DMSO), was added directly to the organoids followed by analysis using live-cell confocal microscopy (Zeiss LSM 800) for 60 minutes at 37°C/5% CO_2. Four technical replicates per patient were included in each experiment (n = 3). The total increase in organoid area relative to t=0 min was calculated using Zen Blue 2.0 analysis software (Zeiss) and results are expressed as area under the curve (AUC) at t=60 minutes.

RNA isolation and gene expression studies

Primer sequences and amplicon sizes are depicted in Supplementary table 2. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Inc), including DNase I treatment of the samples (RNAse-Free DNase Set, Qiagen), according to the manufacturer’s instructions. The purity of RNA was assessed using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) and quantity was measured using the Qubit Fluorometer (Thermo Fisher Scientific) according to the manufacturer’s

instructions. Total RNA (500 ng) of each organoid sample was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.) following the manufacturer's instructions. RT-qPCR reactions to determine *R117H-CFTR* expression were performed on a Bio-Rad CFX96 Real Time Detection System using a two-step RT-qPCR protocol and SYBR Green Supermix (BioRad). Each experiment included standard curves to determine reaction efficiency, together with melting curve analyses to verify amplification specificity and absence of primer dimers. Gene expression was quantified using CFX Manager™ Software (Bio-Rad) qPCR analysis software. Raw *CFTR* cycle of quantification (Cq) values were normalized using *ACTB* and *YWHAZ* housekeeping genes and converted into relative quantities using the R117H-homozygous organoid cell line as reference sample. Three technical replicates per patient were included in each experiment (n=3).

Reference gene selection and validation

To select the most stable reference genes for our RT-qPCR experiments, ten commonly used reference genes were tested based on existing literature [4]: beta-actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-2 microglobulin (*B2M*), glucuronidase beta (*GUSB*), hydroxymethylbilane synthase (*HMBS*), hypoxanthine phosphoribosyltransferase (*HPRT1*), TATA-box binding protein (*TBP*), succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) and ribosomal protein S13 (*RPS13*). Primer sequences and amplicon sizes are shown in Supplementary table 2. RT-qPCR was performed as described above. Individual cDNA samples of eight of the 14 intestinal organoids were used. Reference gene stability and the optimal number of reference genes were evaluated using the web-based tool RefFinder (https://www.heartcure.com.au/reffinder/) and the GeNorm algorithm [5]. The optimal number of reference genes for our experiments was two (GeNorm V coefficient <0.15). The three most stably expressed reference genes in the organoid samples were B2M, YWHAZ and ACTB according to GeNorm (GeNorm M coefficients <0.5) and ACTB, YWHAZ and GUSB according to RefFinder (data not shown). As such, ACTB and YWHAZ were included as reference genes in our RT-qPCR experiments.

R117H-CFTR allele-specific primer design and validation

In supplementary table 2 the (allele-specific) primer sequences are depicted that were used in the R117H-CFTR gene expression studies. A forward primer spanning the exon-exon junction of exons 3 and 4 was designed, together with reverse primers targeting the wild type CFTR sequence or the R117H-CFTR sequence (G→A). In the R117H-CFTR allele-specific reverse primer a deliberate mismatch was introduced

at the penultimate base to enhance reaction specificity using established guidelines [6]. The optimal melting temperature of these primers was determined (62,5˚C) by gradient Q-PCR experiments using cDNA from F508del/R117H and F508del/S1251N organoid cultures. PCR products of allele-specific R117H-CFTR reactions (R117H/R117H organoids) were sequenced to verify the identity of the amplicons. Allele-specific RT-qPCR experiments were performed using similar reaction conditions as described above.

Western blot analysis

Organoids were lysed in Laemmli buffer (2% SDS, 10% glycerol and 63 mM Tris-HCl pH 6.8) supplemented with complete protease inhibitor (Roche, Ltd.). Protein lysates of each sample (50 μg) were used to perform gel electrophoresis, followed by protein transfer to an Immobilon-FL polyvinylidene fluoride (PVDF) membrane (Sigma-Aldrich). The membrane was blocked for 1 hour with 5% w/v milk powder (ELK) dissolved in Tris-buffered saline-Tween (TBST; 0.3% Tween, 10 mM Tris, pH 8, and 150 mM NaCl in H_2O) and incubated o/n at 4°C with primary antibodies mouse α-CFTR 450, 570, 596 (Cystic Fibrosis Folding consortium); 1:5.000 and rabbit α-HSP90 (Braakman laboratory, Utrecht University; 1:50.000), followed by a 1 hour RT incubation with secondary antibodies IRDye 680RD donkey-α-rabbit IgG and IRDye 800CW donkey-α-mouse IgG (LI-COR, Inc.) diluted 1:20.000. All antibodies were diluted in 5% w/v ELK in TBST. Protein detection was performed on the LI-COR Odyssey 3 imaging system (LI-COR, Inc.) using default settings and image resolution. Densitometry analysis was performed using LI-COR Image Studio Lite 5.3 (LI-COR, Inc.). For CFTR protein expression, only mature CFTR protein (C-band) was quantitated. To achieve normalized values, C-band density was corrected for loading using the density of the corresponding HSP90 band and normalized to wild type CFTR protein expression. CFTR protein expression in the organoid cultures was measured in three independent experiments (n=3).

Supplementary table 2. Primer sequences and amplicon sizes.

Gene	Forward primer sequence (5' -> 3')	Reverse primer sequence (5' -> 3')	Amplicon size (bp)	Source
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	117	[a]
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	87	[b]
B2M	CTCCGTGGCCTTAGCTGTG	TTTGGAGTACGCTGGATAGCCT	69	[c]
GUSB	GAAAATATGTGGTTGGAGAGCTCATT	CCGAGTGAAGATCCCCTTTTTA	101	[d]
HMBS	CACGATCCCGAGACTCTGCT	TACTGGCACACTGCAGCCTC	81	[d]
HPRT1	TGAGGATTTGGAAAGGGTGT	GAGCACACAGAGGGCTACAA	118	[d]
TBP	GAGAGTTCTGGGATTGTACCG	ATCCTCATGATTACCGCAGC	143	[e]
SDHA	TGGTTGTCTTTGGTCGGG	GCGTTTGGTTTAATTGGAGGG	85	[e]
YWHAZ	ATGCAACCAACACATCCTATC	GCATTATTAGCGTGCTGTCTT	178	[e]
RPS13	CGAAAGCATCTTGAGAGGAACA	TCGAGCCAAACGGTGAATC	87	[e]
R117H-CFTR	ATTTAGGGGAAGTCACCAAAGCAG	GCCTAGATAAATCGCGATAGATT	107	[f]
CFTR	ATTTAGGGGAAGTCACCAAAGCAG	GCCTAGATAAATCGCGATAGAGC	107	[f]

[a] RtprimerDB: http://www.rtprimerdb.org/
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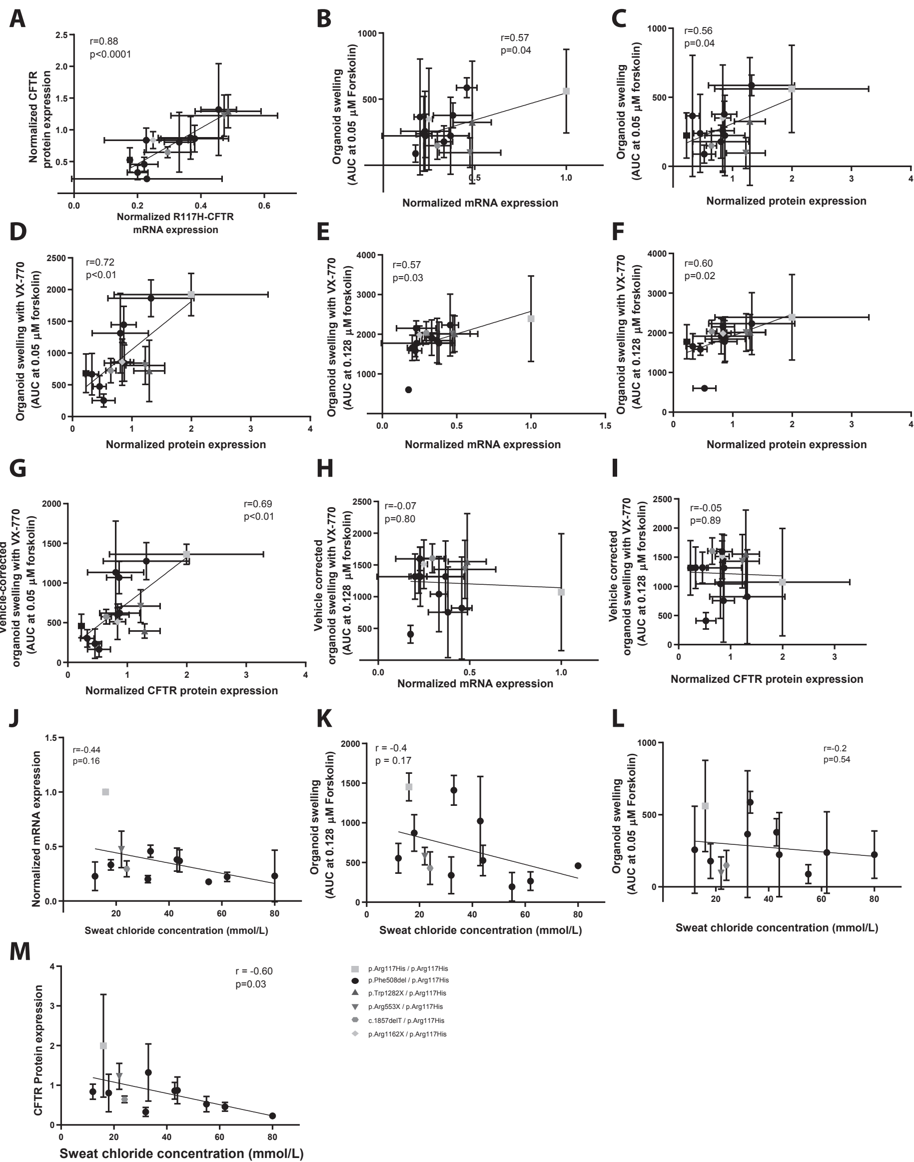
[f] Developed in this study using primer-BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast/ and guidelines published by Wangkumhang *et al.* [6].

Supplementary table 3. Overview of correlation analyses performed in this study.

Variable 1	Variable 2	Pearson's r	p-value
R117H-CFTR mRNA expression	CFTR protein expression	0.92	<0.0001
R117H-CFTR mRNA expression in compound heterozygous R117H cultures	CFTR protein expression in compound heterozygous R117H cultures	0.88	<0.0001
R117H-CFTR mRNA expression	0.128 µm forskolin-induced swelling	0.75	<0.01
R117H-CFTR mRNA expression	0.05 µm forskolin-induced swelling	0.57	0.04
R117H-CFTR mRNA expression	0.128 µm forskolin-induced swelling + 3 µm VX-770	0.57	0.03
R117H-CFTR mRNA expression	0.05 µm forskolin-induced swelling + 3 µm VX-770	0.72	<0.01
R117H-CFTR mRNA expression	0.128 µm forskolin-induced swelling + 3 µm VX-770 corrected for vehicle-treated organoid swelling	-0.07	NS
R117H-CFTR mRNA expression	0.05 µm forskolin-induced swelling + 3 µm VX-770 corrected for vehicle-treated organoid swelling	0.69	<0.01
CFTR protein expression	0.128 µm forskolin-induced swelling	0.74	<0.01
CFTR protein expression	0.05 µm forskolin-induced swelling	0.56	0.04
CFTR protein expression	0.128 µm forskolin-induced swelling + 3 µm VX-770	0.6	0.02
CFTR protein expression	0.05 µm forskolin-induced swelling + 3 µm VX-770	0.72	<0.01
CFTR protein expression	0.128 µm forskolin-induced swelling + 3 µm VX-770 corrected for vehicle-treated organoid swelling	-0.05	NS
CFTR protein expression	0.05 µm forskolin-induced swelling + 3 µm VX-770 corrected for vehicle-treated organoid swelling	0.69	<0.01



Supplementary figure 1. Correlation analyses between FIS, mRNA, and protein expression in R117H-CFTR organoids and sweat chloride concentration. Correlation analyses was performed for (A) normalized R117H-CFTR mRNA expression and normalized CFTR protein expression in compound heterozygous R117H organoids (B) normalized R117H-CFTR mRNA expression and organoid swelling after stimulation with 0.05 µM forskolin, (C) normalized CFTR protein expression and organoid swelling after stimulation with 0.05 µM forskolin, (D) normalized CFTR protein expression and organoid swelling in the presence of 3 µM VX-770 and 0.05 µM forskolin stimulation, (E) normalized R117H-CFTR mRNA expression and organoid swelling in the presence of 3 µM VX-770 and 0.128 µM forskolin, (F) normalized

Supplementary figure 1. Continued.CFTR protein expression and organoid swelling in the presence of 3 μM VX-770 and 0.128 μM forskolin, (G) normalized CFTR protein expression and vehicle-corrected organoid swelling in the presence of 3 μM VX-770 and 0.05 μM forskolin stimulation, (H) normalized CFTR mRNA expression and vehicle-corrected organoid swelling in the presence of 3 μM VX-770 and 0.128 μM forskolin stimulation, (I) normalized CFTR protein expression and vehicle-corrected organoid swelling in the presence of 3 μM VX-770 and 0.128 μM forskolin stimulation, (J) Sweat chloride concentration and normalized R117H-CFTR mRNA expression, (K) Sweat chloride concentration and organoid swelling after stimulation with 0.128 μM forskolin, (L) Sweat chloride concentration and organoid swelling after stimulation with 0.05 μM forskolin and (M) Sweat chloride concentration and CFTR protein expression.

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Potentiator synergy in rectal organoids carrying S1251N, G551D or F508del CFTR mutations

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ABSTRACT

Introduction: The potentiator VX-770 (ivacaftor / KALYDECO™) targets defective gating of CFTR and has been approved for treatment of cystic fibrosis (CF) subjects carrying G551D, S1251N or one of 8 other mutations. Still, the current potentiator treatment does not normalize CFTR-dependent biomarkers, indicating the need for development of more effective potentiator strategies.

Methods: We have recently pioneered a functional CFTR assay in primary rectal organoids and used this model to characterize interactions between VX-770, genistein and curcumin, the latter 2 being natural food components with established CFTR potentiation capacities.

Results: Results indicated that all possible combinations of VX-770, genistein and curcumin synergistically repaired CFTR- dependent forskolin-induced swelling of organoids with CFTR-S1251N or -G551D, even under suboptimal CFTR activation and compounds concentrations, conditions that may predominate *in vivo*. Genistein and curcumin also enhanced forskolin-induced swelling of F508del homozygous organoids that were treated with VX-770 and the prototypical CFTR corrector VX-809.

Discussion: These results indicate that VX-770, genistein and curcumin in double or triple combinations can synergize in restoring CFTR-dependent fluid secretion in primary CF cells, and support the use of multiple potentiators for treatment of CF.

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INTRODUCTION

Cystic fibrosis (CF) is the most common autosomal recessive, life-shortening disorder in the Caucasian population caused by mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene leading to defective CFTR-mediated epithelial ion transport[1,2]. The nearly 2000 *CFTR* mutations that have been identified (www.genet.sickkids.on.ca) so far are categorized into six classes according to their effect on CFTR expression and function[3,4]. The most common mutant CFTR-F508del, expressed by ~90% of all CF individuals, has defects in protein folding and trafficking (class II) and channel gating (class III), and has thermal instability at the apical cell surface (class VI)[5,6]. Classical class III mutations (e.g. G551D and S1251N) affect ~5% of CF subjects and lead to normal apical CFTR processing but severe functional impairment[3] (www.genet.sickkids.on.ca).

Novel therapeutic strategies that target mutation-specific defects of the CFTR protein include repair of CFTR mistrafficking by correctors and defective CFTR channel gating by potentiators[7-10]. The potentiator VX-770 (ivacaftor, KALYDECO™) dramatically improved pulmonary function in subjects with G551D, S1251N or 8 other mutations for which the drug has now been US Food and Drug Administration (FDA)- and European Medicines Agency (EMA)-approved[11-14]. More recently, VX-770 combined with the corrector VX-809/lumacaftor (combination is termed ORKAMBI) showed modest but significant effects on lung function and was FDA-approved for F508del homozygous subjects (approximately 45-50% of all subjects)[15,16]. These studies demonstrate that mutation-specific drug targeting is feasible, but also support the need for more effective treatments, as these treatment do not normalize CFTR-dependent biomarkers for most patients[11-17].

Aside VX-770, many other compounds are able to potentiate CFTR, including the natural food components genistein, an isoflavonoid found in high concentrations in soy[18-20], and curcumin, a major constituent of turmeric[21-23]. Studies have indicated that both VX-770 and curcumin activate CFTR channels in the absence of adenosinetriphosphate (ATP)[21,22,24,25], while genistein is known to promote ATP-dependent CFTR gating[20,26,27]. In line with their different mode of CFTR potentiation, recent patch clamp studies showed additive or even synergistic effects of curcumin and genistein on the gating of G551D-CFTR channels[27,28]. These findings suggest that potentiators with a different mode of action may likewise enhance clinical VX-770 effectiveness.

We recently developed a functional CFTR assay in human intestinal CF organoids[29,30] that was used to study *CFTR* gene-editing[31] and CFTR modulator mechanisms of action[32-34]. One of these studies reported the robust repair of CFTR-F508del trafficking by the combination of structure-guided correctors[33], but data on repair of CFTR gating by potentiator combinations in primary CF cells is lacking. Rapid swelling of organoids induced by forskolin is used to measure the residual and drug-corrected CFTR activity in a subject-specific manner. The robust organoid growth[35-38] and CFTR assay conditions[29,30] allow us to generate large and accurate datasets using drug combinations and forskolin-dose-response curves (Dekkers *et al*. manuscript submitted). FIS was previously shown to be fully CFTR-dependent, as indicated by *CFTR* null mutations and pharmacological CFTR inhibitors[29,33]. We here tested the impact of potentiator combinations on mutant CFTR function in freshly excised rectal biopsies and in organoids generated from these tissues. Results indicated synergy between VX-770, genistein and curcumin in enhancing CFTR-dependent epithelial fluid secretion in a mutation-specific manner, and support the combination of potentiators as therapeutic strategy for CF.

METHODS

Compounds

DMSO stock solutions of VX-770 (20mM; Selleck Chemicals LLC, Houston, USA), genistein (50 mM; Sigma; G6649-25MG) and curcumin (50mM; Fluka; 08511-10MG) were prepared and stored at -80 for a maximum of 6 months. Stock solutions were disposed directly after use and curcumin was protected from light during all procedures.

Human material

Study approval was obtained by the Ethics Committee of the University Medical Centre Utrecht and the Erasmus Medical Centre Rotterdam and informed consent was obtained from all participating subjects. Rectal biopsies were obtained (i) during standard cystic fibrosis care (ii) for diagnostic purposes or (iii) during voluntary participation in studies and used for intestinal current measurements (ICM) and generation of rectal organoids.

Intestinal current measurement (ICM)

Transepithelial, CFTR-dependent anion secretion in human rectal suction or forceps biopsies (in general 4 per subject, for some subjects 1 - 3) was measured using an amendment[56] of the ICM protocol described in detail previously[57]. In short, the biopsies were collected in phosphate-buffered saline on ice and directly mounted

in sliders (aperture 0.011 or 0.018 cm^2) adapted to micro-Ussing chambers (P2400; Physiological Instruments, San Diego, U.S.A.). After equilibration and repetitive prewashing of biopsies, the following compounds were added in a standardized order to the mucosal (M) or serosal (S) side of the tissue: amiloride (100 µM, M) to inhibit amiloride sensitive electrogenic Na^+ absorption; forskolin (10 µM; M+S) to activate CFTR-mediated anion secretion; VX-770 (20 µM, but in some F508del / S1251N biopsies 20 – 40 µM; M+S) to potentiate CFTR; genistein (50 µM, but in 1 F508del / S1251N subject 10 and 100 µM; M+S) to further potentiate CFTR; and carbachol (100 µM; S) to initiate the cholinergic Ca^{2+}- and protein kinase C-linked Cl^- secretion. Crude short circuit current values (µA) were converted to µA cm^{-2} on the basis of the surface area of the aperture. The average response of the biopsies per subject to each addition was used to calculate the group averages +/-SD (Fig.1b,c). An unpaired T-test was used to calculate statistical differences (Fig. 1b).

Crypt isolation and organoid culture from rectal suction biopsies

Methods for crypt isolation and human organoid culturing were slightly adapted from protocols described previously[36]. In short, rectal biopsies were washed with PBS and incubated with 10 mM EDTA for 90 - 120 min at 4 °C. Supernatant was harvested and EDTA was washed away. Crypts were isolated by centrifugation and embedded in 50% matrigel (growth factor reduced, phenol-free, BD bioscience) and seeded (~ 10 - 30 crypts in 3 x 10 µl matrigel droplets per well) in 24-well plates. The matrigel was polymerized for 10 - 30 min at 37 °C and immersed in complete culture medium: advanced DMEM / F12 supplemented with penicillin/streptomycin, 10 mM HEPES, Glutamax, N2, B27 (all from Invitrogen), 1 µM N-acetylcysteine (Sigma) and growth factors: 50 ng ml^{-1} mEGF, 50% Wnt3a-conditioned medium (WCM) and 10% Noggin-conditioned medium (NCM), 20% Rspo1-conditioned medium (RCM), 10 µM Nicotinamide (Sigma), 500 nM A83-01 (Tocris) and 10 µM SB202190 (Sigma). Growth medium was further supplemented with Primocin (1:500; Invivogen). Vancomycin and gentamycin (both from Sigma) were added during the first week of culture.The medium was refreshed every 2–3 days and organoids were passaged 1:4–1:6 every 7–10 days.

The forskolin-induced swelling assay

Methods to measure forskolin-induced organoid swelling described previously[29] were slightly adapted. In short, rectal CF organoids (passage 1–30) from a 7–10-day old culture were seeded in a flat-bottom 96-well culture plate (Nunc) in 5 µl 50% matrigel commonly containing 20–80 organoids immersed in 100 µl complete culture medium. One day after seeding, organoids were incubated for 30 min with 3 µM calcein-green (Invitrogen) in complete culture medium. After calcein-green staining,

forskolin with or without potentiator(s) was added at concentrations as indicated and organoids were directly analyzed by confocal live cell microscopy (LSM710, Zeiss, 5× objective) for 60 min at 37 °C. Two wells were used per condition and experiments were repeated 2–5 times for F508del / S1251N (3 donors) and F508del / F508del (3 donors) organoids and 3–4 times for F508del / G551D organoids (1 donor).

Quantification of forskolin-induced swelling

Forskolin-stimulated organoid swelling was automatically quantified using Volocity imaging software (Improvision). The total organoid area (xy plane) increase relative to t = 0 of forskolin treatment was calculated. In some cases, cell debris and unviable structures were manually excluded based on criteria described in detail in a standard operating procedure (SOP). The area under the curve (AUC; t = 60; baseline = 100%) was calculated using Graphpad Prism. An unpaired T-test was used to calculate statistical differences (Fig. 2 - 4).

Western blot analysis

Organoids with S1251N or G551D from a 7 day-old culture were passaged 1:1 in 24-well plates and incubated with DMSO, VX-770, genistein, curcumin or combinations (1 well per condition) in 0.5 ml complete growth medium for 48 hours. The medium with compounds was refreshed after 24 h. Cells were lysed in Laemmli buffer supplemented with complete protease inhibitor tablets (1:50; Roche). Lysates were analyzed by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% milk protein in TBST (0.3% Tween, 10 mM Tris pH8 and 150 mM NaCl in H_2O) and probed 3 h at RT with mouse monoclonal E-cadherin-specific (1:10000; DB Biosciences) or CFTR-specific antibodies (450, 570 and 596; 1:5000; Cystic Fibrosis Folding consortium), followed by incubation with HRP-conjugated secondary antibodies (1:3000) and ECL development. An unpaired T-test was used to calculate statistical differences (Fig. 2k and 3j).

RESULTS

Intestinal current measurements of F508del / S1251N rectal biopsies treated with VX-770 and genistein

Because different CFTR activation mechanisms for VX-770 (ATP-independent) and genistein (ATP-dependent) have been described[20,24-27], we first assessed treatment of VX-770, genistein and their combination by intestinal current measurements (ICM) on human rectal biopsies derived from 17 F508del homozygotes or 7 compound heterozygotes expressing F508del and the gating mutation S1251N (Fig. 1). We

observed a chloride secretory response to forskolin, which was ~5 times greater in F508del / S1251N compared to F508del / F508del biopsies (Fig. 1a,b). In biopsies expressing S1251N, the response to forskolin was 34% ± 13.1 (mean ± SD) of the average response of 43 healthy controls measured in our centre (Δ Isc of 57 μA/cm^2; data not shown), indicating a detection window for potentiator treatment. We observed that that response to forskolin in biopsies with S1251N was increased by VX-770 and even further enhanced by genistein, while potentiator activity was not detected in F508del homozygous biopsies (Fig. 1a-b). To conclude, these data indicate that ICM can detect potentiator activity on CFTR-S1251N, but not on CFTR-F508del, and that treatment of VX-770 and genistein together is more effective than of VX-770 alone.

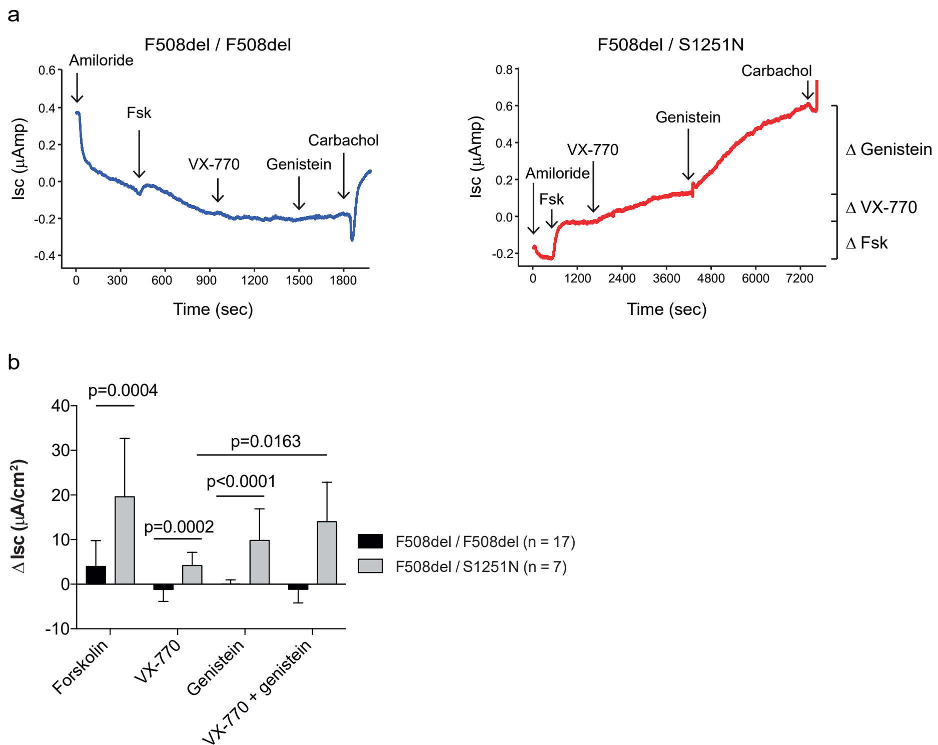

Figure 1. Intestinal current measurements of F508del / S1251N rectal biopsies treated with VX-770 and genistein. (a) Examples of intestinal current measurement (ICM) tracings of a F508del / F508del or F508del / S1251N rectal biopsy incubated with compounds as indicated (amiloride 100 μM; forskolin (fsk) 10/100 μM; VX-770 20 μM; genistein 50 μM; carbachol 100 μM). (b) Delta Isc ICM values of biopsies derived from 17 F508del / F508del (2–4 biopsies per subject) and 7 F508del / S1251N subjects (1–4 biopsies per subject).

Incubation of VX-770, genistein and curcumin in rectal organoids derived from F508del / S1251N CF subjects

To investigate interactions between different potentiators at larger scale, we analyzed effects of VX-770, genistein, curcumin and their combinations on forskolin-induced swelling (FIS) of organoids derived from CF subjects compound heterozygous for F508del and the gating mutation S1251N. As described previously[29], organoid swelling was presented as absolute area under the curve (AUC) calculated from 60 min time tracings of the surface area increase of calcein-green-labeled organoids relative to t = 0 (Fig. 2a,b). We assessed titrations of VX-770, genistein and curcumin using suboptimal forskolin concentrations (0.128 and 0.5 μM) (Fig. 2c-e) to remain within the dynamic range of the swelling assay (Fig. 2f,g) and to facilitate an optimal detection of potentiator activity. In line with other studies[28] (Dekkers *et al*. manuscript submitted), we consistently observe that the FIS assay reaches it's upper detection limit around 3000-3500 AUC units, most likely because basolateral ion transport becomes rate-limiting above a certain threshold of CFTR function. We observed that all compounds dose-dependently increased FIS of organoids, with highest potency for VX-770 and lowest potency for curcumin (Fig. 2c-e).

Next, we studied VX-770 and genistein combination therapy using a forskolin dose-range (0.008 - 5 μM) (Fig 2f,g) and observed that near-saturating (3 or 50 μM; Fig. 2f) and suboptimal (0.1 or 10 μM; Fig. 2g) concentrations of VX-770 or genistein increased basal FIS in a forskolin dose-dependent manner. Interestingly, genistein greatly enhanced VX-770-repaired FIS and synergistic effects were observed at low forskolin concentrations (Fig 2f,g). As observed previously (Dekkers *et al*. manuscript submitted), the drug-induced FIS of S1251N-expressing organoids reached maximal rates (= AUC of ~3000) at high forskolin levels (Fig 2f,g). The genistein dose-dependent increase in FIS of VX-770-treated organoids also indicated synergy between VX-770 and genistein, even at genistein levels <10 μM (Supplementary Fig. S1a). Subsequently, combinations of VX-770, genistein and curcumin were investigated at near-saturating (Fig. 2h; 3, 50 and 50 μM) and suboptimal (Fig. 2i; 0.1, 10 and 10 μM) potentiator dosages using fixed forskolin concentrations, which were defined from Fig. 2f,g. At near-saturating potentiator concentrations, most FIS responses upon combination treatments (except for genistein + curcumin) greatly exceeded the calculated additive values of the single treatments, indicating synergistic effects for these potentiator combinations (Fig. 2h). Stimulations with suboptimal potentiator concentrations indicated a strong increase of the VX-770 response by genistein, but not by curcumin, albeit that synergy was detected for the

triple combination treatment, but not for the VX-770 + genistein condition (Fig. 2i) . In line with Fig. 2h and 2i, the response of VX-770- or genistein-treated organoids was dose-dependently increased by curcumin and appeared more prominent at higher curcumin concentrations (>12.5 μM; Supplementary Fig. S1b,c). Potentiator-induced swelling was absent in organoids expressing two class I *CFTR* mutations, indicating that the FIS induced by VX-770, genistein and curcumin is fully dependent on function of CFTR (Supplementary Fig. S2). We also assessed CFTR-S1251N protein expression detected by Western blot upon chronic stimulation (48 h) of VX-770, genistein, curcumin or combinations (Fig. 2j,k). Results indicated that the VX-770 + curcumin and triple combination treatments reduced expression of maturated CFTR C-band to ~70% of mock-treated organoids (Fig. 2k). In conclusion, all potentiator combinations synergistically repaired CFTR- dependent epithelial fluid transport in organoids with CFTR-S1251N, suggesting that VX-770, genistein and curcumin potentiate CFTR by different mechanisms. The synergy was detected at suboptimal activation of CFTR and suboptimal potentiator levels, conditions that may predominate *in vivo*.

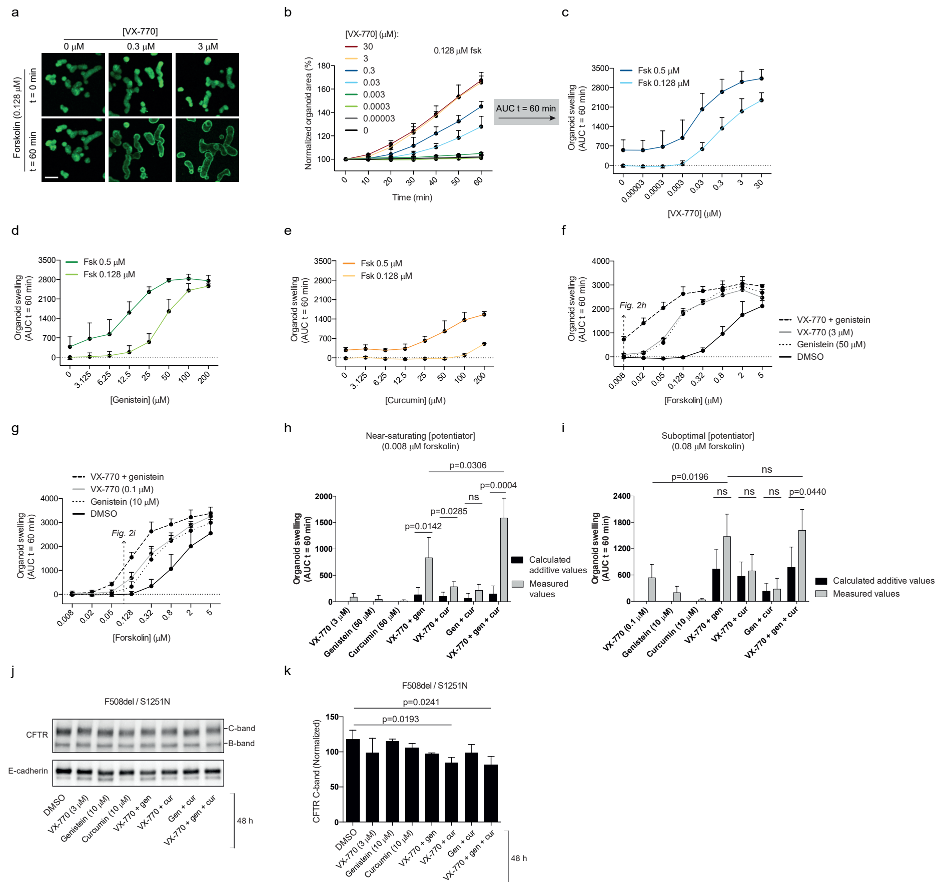

Figure 2. Incubation of VX-770, genistein and curcumin in rectal organoids derived from F508del / S1251N CF subjects. (a) Representative confocal images of calcein-green-labeled F508del / S1251N organoids at the indicated time points of forskolin (0.128 µM) stimulation. Scale bar = 130 µm. (b) A time tracing of the forskolin(0.128 µM)-induced surface area increase relative to t = 0 (normalized area) of F508del / S1251N organoids at different VX-770 concentrations averaged from two independent wells. Mean ± SD. (c-e) Forskolin(fsk)-induced swelling (FIS) expressed as the absolute area under the curve (AUC) calculated from time tracings shown in b (baseline = 100%, t = 60 min) of organoids stimulated with multiple dosages of VX-770 (c), genistein (d) or curcumin (e) at the indicated forskolin concentrations. (f,g) FIS of organoids stimulated with near-saturating (f) or suboptimal (g) concentrations of VX-770, genistein or both using a dose range of forskolin. (h,i) FIS of organoids stimulated with near-saturating (h) or suboptimal (i) concentrations of VX-770, genistein (gen), curcumin (cur) or their combinations at the indicated concentrations of forskolin, corrected for the FIS without addition of a potentiator. (c-i represent data averaged from 3 F508del / S1251N subjects. Each subject was measured at 2 to 5 independent culture time points in duplicate. Mean ± SD. The SD indicates the inter-subject variation). (j) Expression of CFTR and E-cadherin in whole cell lysates of F508del / S1251N organoids detected by Western blot upon treatments as indicated (48 h). Immature (B-band) and mature (C-band) CFTR is indicated. (h) Quantification of CFTR C-band by Image J from Western Blots as shown in j. Data were normalised to the average of the 8 conditions per experiment. Data of 3 different F508del / S1251N cultures were averaged, each culture was assessed in 1-3 independent experiments. P values are indicated only for the conditions that were statistically different from the DMSO control. Mean ± SD.

Incubation of VX-770, genistein and curcumin in rectal organoids derived from an F508del / G551D CF subject

We next analyzed effects of VX-770, genistein, curcumin and their combinations on FIS of F508del / G551D organoids (Fig. 3) using an experimental setup comparable to Fig. 2. Because CFTR-S1251N has a higher residual activity than CFTR-G551D (Fig. 2f,g and 3e,f), different forskolin concentrations are required for optimal drug testing in F508del / S1251N and F508del / G551D organoids (Fig. 2 and Fig. 3). Titrations of VX-770, genistein and curcumin indicated a dose-dependent increase in FIS of F508del / G551D organoids, with highest potency for VX-770 and lowest potency for curcumin (Fig. 3a-c), similar as observed for F508del / S1251N organoids (Fig. 2c-e). VX-770 and genistein at near-saturating (Fig. 3d,e) or suboptimal (Fig. 3f) concentrations synergistically repaired FIS of F508del / G551D organoids at suboptimal forskolin levels. Compared to S1251N-expressing organoids (Fig. 2f,g), optimal detection of synergy between VX-770 and genistein in F508del / G551D organoids required somewhat higher forskolin levels, probably because of the low residual function associated with CFTR-G551D (Fig. 3e,f). Furthermore, both VX-770 and genistein induced FIS to a similar extent in S1251N-expressing organoids (Fig. 2f,g), while the effect of genistein was much lower compared to VX-770 in G551D-expressing cultures (Fig. 3e,f). This suggests that channel gating by genistein, but not by VX-770, is critically dependent on the site of the class III mutation within the multi-domain structure of the CFTR channel. We selected optimal forskolin concentrations based on Fig. 3e,f to study the effect of curcumin in addition to VX-770 and genistein using near-saturating (Fig. 3g) or suboptimal (Fig. 3h) potentiator concentrations and observed strong synergy for most double and triple combinations, except for genistein + curcumin at saturating dose (Fig. 3g,h). In contrast to F508del / S1251N organoids (Fig. 2i), curcumin, in combination with VX-770, was highly effective at suboptimal dose in organoids with G551D (Fig. 2h), suggesting that CFTR-G551D is more sensitive to curcumin than CFTR-S1251N. Chronic incubation of potentiators revealed that CFTR C-band expression was only reduced by genistein and curcumin (~70% of vehicle Fig. 3i,j). To conclude, organoids expressing CFTR-S1251N or -G551D responded differently to identical potentiator levels and CFTR phosphorylation conditions, suggesting that different molecular mechanisms underlie the gating defects of these mutants.

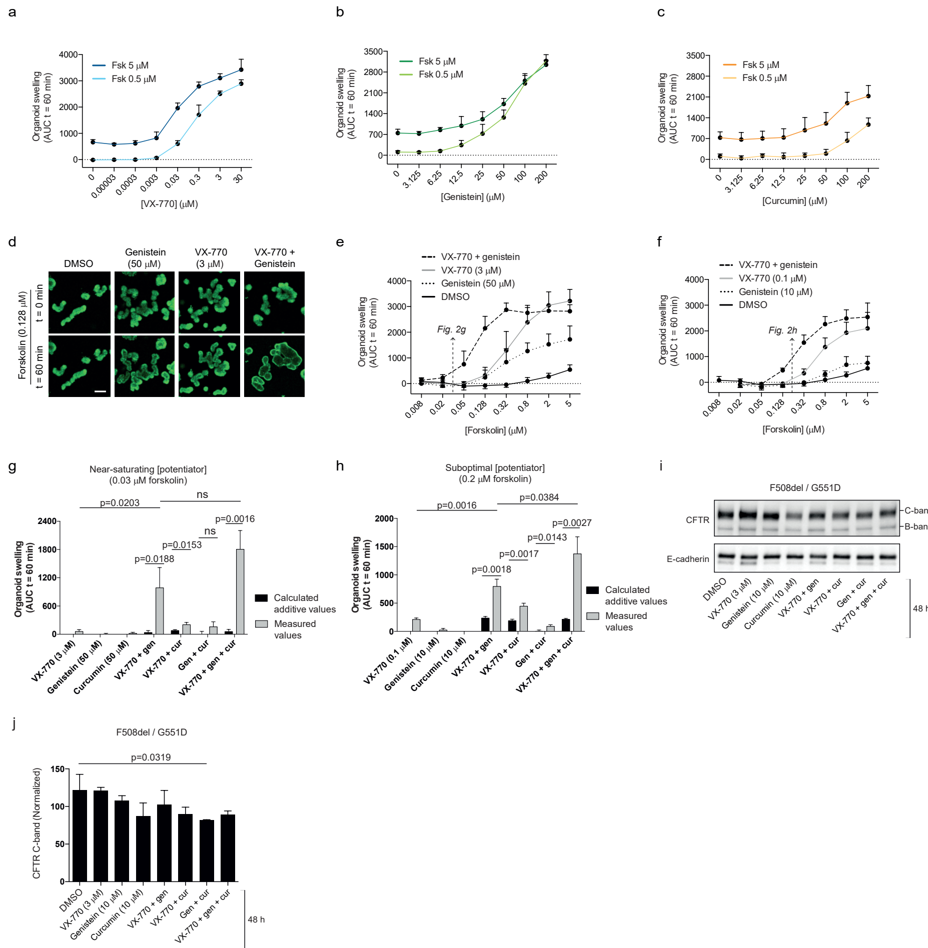

Figure 3. Incubation of VX-770, genistein and curcumin in rectal organoids derived from a F508del / G551D CF subject. (a-c) Forskolin(fsk)-induced swelling (FIS) expressed as the absolute area under the curve (AUC) calculated from time tracings shown in Fig. 2b (baseline = 100%, t = 60 min) of organoids stimulated with multiple dosages of VX-770 (a), genistein (b) or curcumin (c) at the indicated forskolin concentrations. (d) Representative confocal images of calcein-green-labeled organoids at the indicated time points of forskolin (0.128 μM) stimulation. Scale bar = 130 μm. (e,f) FIS of organoids stimulated with near-saturating (e) or suboptimal (f) concentrations of VX-770, genistein or both using a dose range of forskolin. (g,h) FIS of organoids stimulated with near-saturating (g) or suboptimal (h) concentrations of VX-770, genistein (gen), curcumin (cur) or their combinations at the indicated concentrations of forskolin, corrected for the FIS without addition of a potentiator. (a-c and e-h represent data from 1 F508del / G551D subject measured at 3 to 4 independent culture time points in duplicate. Mean ± SD. The SD indicates the inter-experiment variation). (i) Expression of CFTR and E-cadherin in whole cell lysates of F508del / G551D organoids detected by Western blot upon treatments as indicated (48 h). Immature (B-band) and mature (C-band) CFTR is indicated. (j) Quantification of CFTR C-band by Image J from Western Blots as shown in i. Data were normalised to the average of the 8 conditions per experiment. Data of 3 independent experiments was averaged. P value is indicated only for the condition that is statistically different from the DMSO control Mean ± SD.

Incubation of VX-770, genistein and curcumin in rectal organoids derived from F508del / F508del CF subjects

Direct stimulations by VX-770, genistein and curcumin were also assessed in organoids homozygous for the most common *CFTR* mutation F508del. The hierarchy of the compound potency (VX-770 > genistein > curcumin) was similar as observed for organoids with a gating mutation (Fig. 2c-e and 3a-c), but potentiator-induced FIS rates were much lower in F508del homozygous organoids, even when a saturating forskolin dose was used (5 µM) (Fig. 4a-c). Synergy between VX-770 and genistein was observed using near-saturating potentiator dosages (0.05 - 0.8 µM forskolin; Fig. 4d) and VX-770 and genistein additively increased FIS using suboptimal dosages (0.32 – 2 µM forskolin; Fig. 4e). Importantly, genistein greatly enhanced FIS of organoids that were treated with VX-770 or VX-770 and VX-809, even at genistein levels < 10 µM (Fig. 4f and Supplementary Fig. S3a,b). In VX-809-corrected organoids, using saturating (3 and 50 µM; Fig. 4g) and suboptimal (0.1 and 10 µM; Fig. 4h) potentiator concentrations, we detected synergy between VX-770 and genistein at near-saturating dose, but not for any other combination (Fig. 4g,h). Western blot assays were performed to assess the effects of chronic stimulations on protein levels, but the lack of robust detection of a CFTR-F508del C-band prevented proper quantification and interpretation of the results (Fig. S3c). In conclusion, these data indicate that FIS of VX-809-corrected or non-corrected CFTR-F508del homozygous organoids is synergistically repaired by VX-770 and genistein, but not by curcumin.

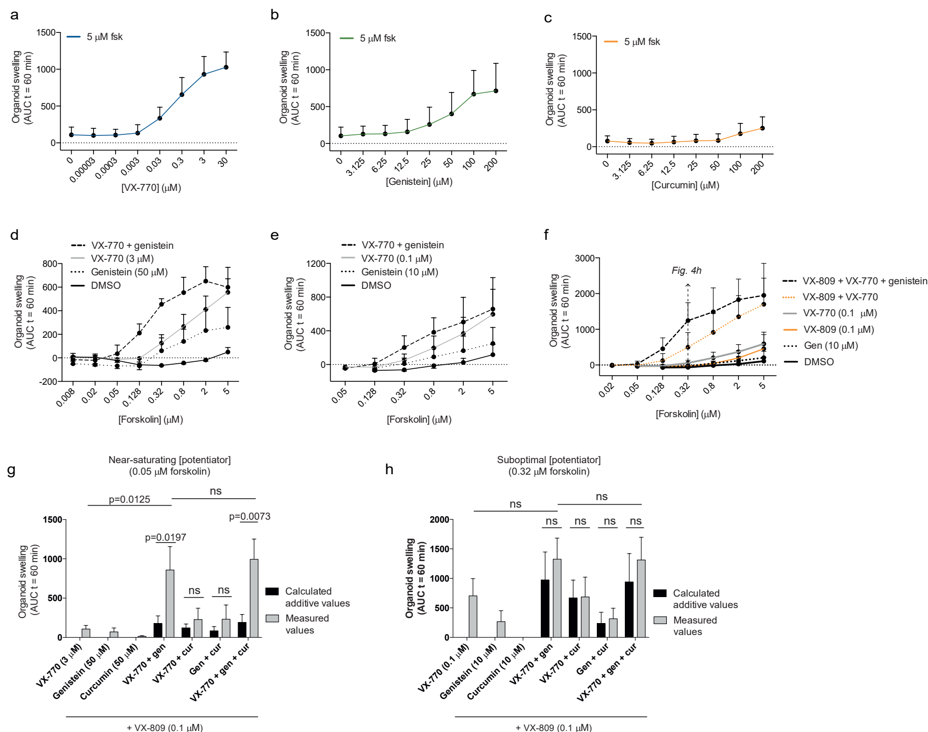

Figure 4. Incubation of VX-770, genistein and curcumin in rectal organoids derived from F508del / F508del CF subjects. (a-c) Forskolin(fsk)-induced swelling (FIS) expressed as the absolute area under the curve (AUC) calculated from time tracings shown in Fig. 2b (baseline = 100%, t = 60 min) of organoids stimulated with multiple dosages of VX-770 (a), genistein (b) or curcumin (c) at the indicated forskolin concentrations. (d,e) FIS of organoids stimulated with near-saturating (d) or suboptimal (e) concentrations of VX-770, genistein or both using a dose range of forskolin. (f) The effect of VX-809 preincubation (24 h) on FIS of organoids stimulated with VX-770 or genistein using a dose range of forskolin. (g,h) FIS of organoids pre-incubated with VX-809 for 24h, and stimulated with near-saturating (g) or suboptimal (h) concentrations of VX-770, genistein (gen), curcumin (cur) or their combinations at the indicated concentrations of forskolin, corrected for the VX-809-repaired FIS without addition of a potentiator. (All figures represent data averaged from 3 F508del / F508del subjects. Each subject was measured at 2 to 5 independent culture time points in duplicate. Mean ± SD. The SD indicates the inter-subject variation).

DISCUSSION

We characterized interactions between VX-770 (ivacaftor, KALYDECO^{TM}) and the natural food components genistein and curcumin using rectal CF biopsies and primary intestinal CF organoids as *ex vivo* models. While CFTR repair by corrector / corrector or corrector / potentiator combinations has been abundantly examined[15,29,33,39-41], only a few studies have reported interactions between potentiators[28,39], and similar studies in primary cells including VX-770 are completely lacking. In this study, we

showed for the first time that (i) the effectiveness of the only clinically available potentiator VX-770 can be greatly enhanced by genistein and curcumin, (ii) combining 3 potentiators enhances CFTR-dependent epithelial fluid secretion to a greater extent compared to the combination of 2 potentiators, and (iii) potentiator combinations exert synergistic effects in a *CFTR* mutation-specific fashion. These data support the development of potentiator combination therapy in clinical practice, especially for people expressing gating mutations.

In line with previous studies[42], we observed that potentiator activity is detected by ICM in rectal biopsies expressing a CFTR gating mutation (Fig. 1). However, while we did observe a chloride secretory response to forskolin in F508del homozygous biopsies of some CF patients, a further stimulation by CFTR potentiators appeared marginal or absent (Fig. 1a,b). In contrast, the organoid assay (FIS) allowed clear detection of both residual function and potentiator activity, in comparison to ICM. Since ICM was able to detect limited residual function in F508del homozygous biopsies, it appears that especially potentiator delivery is difficult in the fresh rectal biopsies. Organoid measurements furthermore allow generation of large subject-specific datasets with forskolin and potentiator dose-ranges and inter-experimental variance, while ICM is in general restricted to maximal 4 biopsies. Compared to stable drug responses of organoids that can be generated for the individual (Dekkers *et al*. Manuscript submitted), we have experienced greater variability between different ICM measurements (Fig. 1c) as well as between different biopsies and the limited amount of biopsies severely hinders the inclusion of e.g. control stimulation (data not shown). On the other hand, ICM is one of the few techniques capable of measuring CFTR activity in native epithelium *ex vivo*, is completely free of potential cell culture artifacts, and can be used as an *ex vivo* biomarker to study drug effectiveness *in vivo*[43,44]. Clearly, both methods are complementary and need to be validated further as diagnostic, prognostic, and therapeutic biomarkers.

Opening of the CFTR gate is regulated by phosphorylation of the regulatory(R)-domain and ATP-dependent dimerization of the nucleotide binding domain(NBD)1 and NBD2. Intriguingly, both VX-770 and curcumin activate CFTR channels in the absence of ATP, suggesting that they bind directly to the channel pore and bypass the conventional ATP-dependent gating mechanism[21,22,24,25]. In contrast, genistein is known to promote ATP-dependent gating of CFTR, probably by binding to the NBD2 and/or the NBD1-NBD2 interface and inhibiting ATP hydrolysis[20,26,27]. As expected from their different mode of potentiation and previous findings[27,28], combinations of genistein with VX-770 or with curcumin synergistically repaired FIS of organoids carrying the “pure” gating mutations S1251N and G551D (Fig. 2 and 3). Remarkably,

however, we also observed synergy between curcumin and VX-770, suggesting that the binding sites for these potentiators, or their mechanism of ATP-independent gating are not identical either. Data of rectal biopsies (Fig. 1) and organoids (Fig. 2h,i; 3g,h) indicate that at near-saturating concentrations of VX-770 the FIS of S1251N- and G551D-expressing cultures can be further enhanced by other potentiators, in particular by genistein. This supports previous observations in other G551D-expressing cell models[8,25] showing that the open probability (*Po*) of VX-770-treated G551D-CFTR remains far below the *Po* of wild-type CFTR channels. On the basis of these *in vitro* findings we have recently initiated clinical studies to investigate *in vivo* effects of genistein supplementation in Dutch CF subjects with the S1251N mutation that are treated with VX-770.

Albeit that VX-770, genistein and curcumin are supposed to improve forskolin-induced swelling in organoids principally through the direct repair of defective CFTR channel gating, these compounds may influence the epithelial fluid transport via other mechanisms as well. Organoid swelling as a measure of CFTR activity is dependent on several other, potential rate-limiting steps in transepithelial fluid transport, e.g. the import of chloride and bicarbonate at the basolateral membrane, and the activity of hyperpolarizing basolateral potassium channels which, in concert, dictate the electrochemical driving force for anion exit across the apical CFTR channel. Therefore, though the fluid secretion is completely CFTR-dependent and proportional to CFTR activity within the dynamic range of the assay (Dekkers *et al*, manuscript submitted), compounds or conditions that affect the driving force for anion exit and/or co-determine the phosphorylation state of CFTR in the presence of forskolin (e.g. phosphodiesterase or protein phosphatase inhibitors), may also have an impact on the FIS assay. On one hand this consideration complicates the mechanistic interpretation of pharmacological stimulation of organoid swelling in case the stimulus is not just a pure CFTR potentiator. On the other hand the FIS assay measures restoration of net fuid secretion and luminal fluidity in primary epithelial organoids under quasi-physiological conditions, which is more relevant for CF patients than CFTR activity in isolation, as measured in patch clamp or iodide efflux studies.

Previous studies report that the functional response of CFTR-wild-type and -F508del channels to genistein is bell-shaped, i.e. enhanced in a low concentration range and inhibited in a higher concentration range, suggesting the existence of a high affinity activatory binding site and a low affinity inhibitory binding site in CFTR[45,46]. The inhibitory effect of genistein was not observed for CFTR-G551D, most likely because the G551D mutation abolishes the low affinity inhibitory binding site[20,47], which argues

for the therapeutic use of genistein in G551D-expressing subjects. Remarkably, our results indicated that genistein (up to 200 µM) dose-dependently and uniformly increased FIS of organoids for all three mutants investigated, i.e. G551D, S1251N and F508del, without signs of inhibition at the highest dose (Fig. 2d, 3b and 4b). Possibly, the inhibitory effect of genistein on CFTR-F508del depends on the cell model used, or the genistein concentration that reaches CFTR in organoids is lower than the concentration in the surrounding medium. Compounds need to penetrate the matrigel that serves as 3D support, enter via the basolateral epithelial membrane and diffuse to the apical site to reach CFTR, which may differentially impact the efficacy of CFTR modulators. This may also explain why curcumin mono treatment of CFTR-G551D and –S1251N organoids was only effective at 25 µM and higher, while a higher CFTR-activating potency of curcumin was observed in other studies[21,28].

Aside the general similarities in the behavior of the S1251N and G551D gating mutants in the FIS assay and in their response to the potentiators noted above, pronounced differences were found in their basal activity (S1251N > G551D; Fig. 2f,g vs. 3e,f), in their maximal response to genistein (S1251N > G551D; Fig. 2f vs. 3e), and in their sensitivity to curcumin in the presence of VX-770 and genistein (G551D > S1251N; Fig. 3h vs. 2i). At a first glance these differences are rather unexpected because both mutated residues are situated inside the functionally important ATP binding pocket 2 (ABP2) of CFTR, and are predicted to disrupt ATP-induced head-to-tail dimerization of NBD1 and NBD2. However, residue G551 is part of the ABC signature sequence of NBD1, which is involved in ATP hydrolysis rather than ATP binding[48], and the G551D mutation has been shown to abolish ATP hydrolysis and to convert ABP2 from a stimulatory into an inhibitory site[49]. Instead, residue S1251 is located in the Walker A sequence of NBD2 which is crucial for ATP binding[48], suggesting that the S1251N mutation most plausibly impairs ATP binding at site 2 rather than affecting ATP hydrolysis or creating an ATP-inhibitory site. Such functional differences may have a strong impact on the open probability of the mutant CFTR channels and on their differential response to the potentiators. Clearly, additional studies, in particular of the poorly explored S1251N mutant channels, are needed to improve our mechanistic understanding of these differences.

Previous studies indicated that chronic stimulation of potentiators may diminish expression and functionality of VX-809-repaired CFTR-F508del or wild-type CFTR, but not of CFTR-G551D, by yet undefined mechanisms[50,51]. In line with these studies, chronic stimulation with VX-770 did not affect protein expression of CFTR-G551D in organoids (Fig. 3i,j). While chronic single potentiator treatment had no effect on the protein expression of CFTR-G551D or CFTR-S1251N, some potentiatior

combinations triggered modest reduction in the protein levels of these gating mutants (Fig. 2j,k and 3i,j). Whether a similar reduction in CFTR protein expression occurs upon chronic treatment of F508del organoids is difficult to evaluate, considering the very low intensity of the F508del-CFTR band C in VX-809-pretreated organoids even in the absence of any potentiator (Supplementary Fig. S3c). Moreover, functional studies of CFTR after 48 h of chronic potentiator treatment by the FIS assay are difficult to perform as the chronic presence of a potentiator induces organoid swelling in the absence of forskolin, which by itself suggest increased functional activity (Dekkers *et al*. manuscript submitted). Because it is impossible to exactly mimic chronic potentiator treatment *in vitro* in terms of temporal concentrations reaching CFTR *in vivo*, the clinical implications of small potentiator-dependent reductions in CFTR-S1251N and -G551D C-band levels observed *in vitro* are hard to predict. However, clinical studies with F508del homozygotes indicated that combination treatment with VX-809 and VX-770[15,16] is more effective than VX-809 treatment alone[52], suggesting that positive effects on CFTR gating by potentiator combinations likely outweigh possible CFTR destabilizing effects.

Previous studies[29] (Dekkers *et al.* manuscript submitted) already indicated that maximal FIS rates (~3000 AUC units) can be reached at higher forskolin levels, most likely because the basolateral chloride import, and not the apical CFTR function, becomes rate limiting. Therefore, optimal detection of synergy between the potentiators in organoids carrying the S1251N or G551D mutations required a titration of forskolin (Fig. 2 and 3). In general, the residual and potentiator-induced FIS levels of F508del homozygous organoids (Fig. 4) were greatly reduced compared to organoids generated from compound heterozygotes carrying both F508del and a gating mutation (Fig. 2 and 3), indicating that FIS responses in these organoids mainly reflect the activity of CFTR-S1251N (Fig. 2) or -G551D rather than -F508del (Fig. 3).

Performing the FIS assay on rectal organoids generated from F508del homozygous patients allowed several important conclusions (Fig. 4): (i) even at saturating forskolin concentrations (5 μM) the response to the potentiators genistein and VX-770 could be increased further by the corrector VX-809, suggesting that maximal potentiation is rate limited by low CFTR-F508del protein levels; (ii) the ranking order of the compound potency (VX-770 > genistein > curcumin) was similar as observed for organoids with a gating mutation (Fig. 2c-e and 3a-c); and (iii) genistein greatly enhanced FIS of F508del organoids that were treated with VX-770 (Fig. 4g),. As these responses are observed upon direct stimulation of the compounds, they rather

act on limited amounts of available surface CFTR-F508del than promoting CFTR-F508del trafficking.

Aside their action as CFTR potentiators we focussed on above, both genistein and curcumin possess various other biological effects (not reported as yet for VX-770) including antioxidation, antiproliferation and anticarcinogenic, and many clinical trails have been performed to study their clinical efficacy as mono-treatment for various diseases. The low oral bioavailability of both compounds due to poor absorption and rapid metabolism may relate to the ambiguous therapeutic effects and large inter-subject variation observed in these studies[53-55]. Pharmacokinetic studies indicated that plasma levels of active compound can be reached in the nM range for curcumin and μM range for genistein, conditions that induced only limited clinical effects in mono-therapy studies[53,54]. However, the strong synergistic potentiator effects observed here suggest that even low curcumin and genistein plasma levels may be sufficient to functionally repair CFTR-dependent fluid secretion either by duo-treatment or triple-treatment including VX-770, especially if drugs would further accumulate in the affected tissues.

In conclusion, functional CFTR measurements in rectal CF tissue support that (i) organoid measurements can detect potentiator activity more sensitive and robustly compared to ICM, (ii) VX-770, curcumin and genistein have different mechanisms of CFTR potentiation, (iii) the gating defects in CFTR-S1251N, -G551D and F508del are functionally different from each other, (iv) acute addition of potentiator combinations at suboptimal or near-saturating dosage synergistically repaired gating of CFTR-S1251N and -G551D and to a lesser extent of VX-809-repaired CFTR-F508del, (v) stimulating interactions between different potentiators are detected within a wide range of forskolin concentrations and (vi) expression of CFTR-S1251N and CFTR-G551D are modestly affected some chronic potentiator combination treatments. These results highlight the potential of combining potentiators for the therapy of Cystic Fibrosis.

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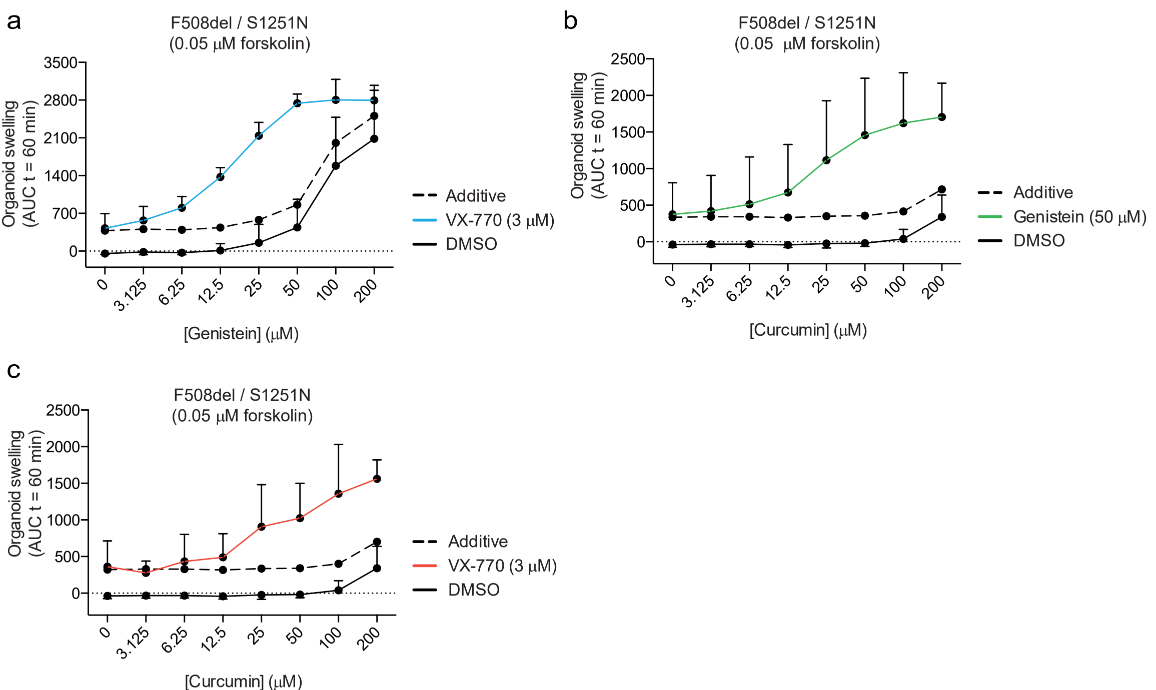
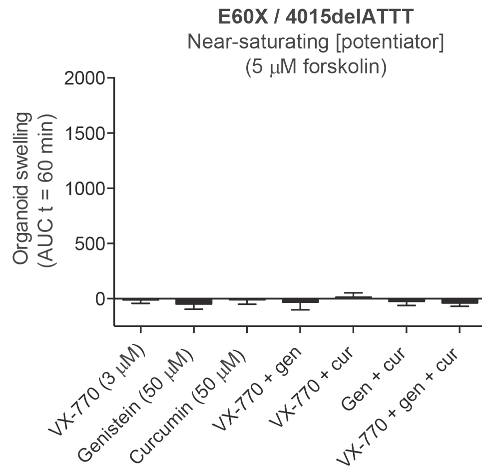
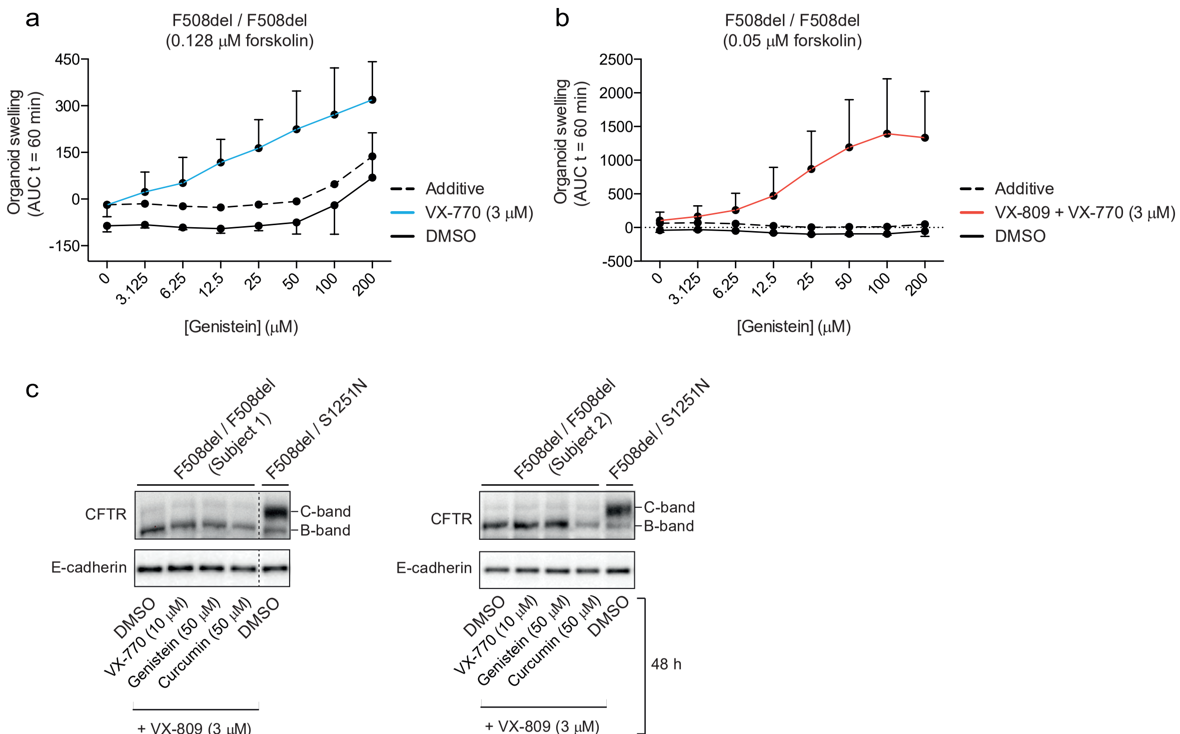
SUPPLEMENTARY DATA



Supplementary figure S1. Dose-dependent potentiator synergy in F508del / S1251N organoids. (a-c) Forskolin-induced swelling of F508del / S1251N organoids stimulated with a dose range of genistein with or without VX-770 treatment (a), or stimulated with a dose range of curcumin with or without genistein (b) or VX-770 (c) at the indicated concentration of forskolin (this concentration was selected using Fig. 2f). The calculated additive responses of the single treatments are indicated as a dashed line. (a-c represent data averaged from 3 F508del / S1251N subjects. Each subject was measured at 2 to 5 independent culture time points in duplicate. Mean ± SD. The SD indicates the inter-subject variation).



Supplementary figure S2. Incubation of VX-770, genistein and curcumin in rectal organoids derived from a CF subject expressing two class I CFTR mutations. Forskolin-induced swelling (FIS) of organoids stimulated with near-saturating concentrations of VX-770, genistein (gen), curcumin (cur) or combinations at a saturating concentration of forskolin, corrected for the FIS without addition of a potentiator. The subject was measured at 2 independent culture time points in duplicate. Mean ± SD.



Supplementary figure S3. Dose-dependent potentiator synergy in F508del / F508del organoids. (a,b) Forskolin-induced swelling of F508del homozygous organoids stimulated with a dose range of genistein with or without VX-770 (a) or VX-770 + VX-809 (b) at the indicated concentrations of forskolin. The calculated additive responses of the single treatments are indicated as a dashed line. (a and b represent data averaged from 3 F508del / F508del subjects. Each subject was measured at 2 independent culture time points in duplicate. Mean ± SD. The SD indicates the inter-subject variation). (c) Expression of CFTR and E-cadherin in whole cell lysates of F508del / F508del (2 different donors) and F508del / S1251N organoids detected by Western blot upon treatments as indicated (48 h). Of note, higher compound concentrations were used as compared to Fig. 2j,k and 3i,j. Immature (B-band) and mature (C-band) CFTR is indicated.

Protocol for application, standardization and validation of the forskolin induced swelling assay in Cystic Fibrosis human colon organoids

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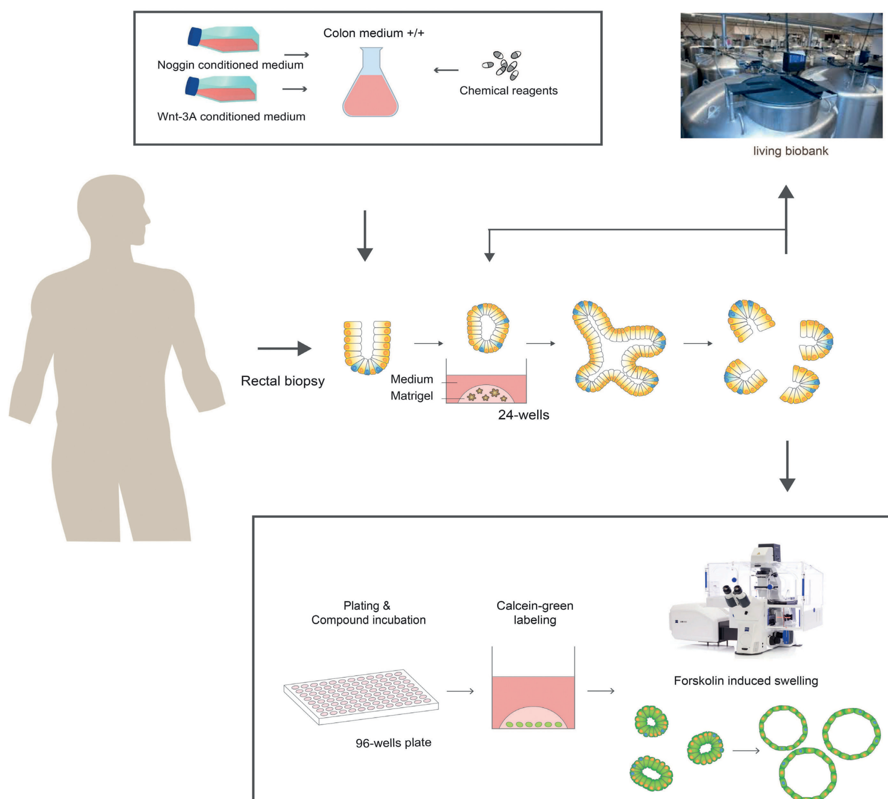
SUMMARY

This protocol describes the isolation, handling, culture of and experiments with human colon stem cell organoids in the context of cystic fibrosis (CF). In human colon organoids, the cystic fibrosis transmembrane conductance regulator (CFTR) protein function and its rescue by CFTR modulators can be quantified using the forskolin induced swelling assay. Implementation procedures and validation experiments are described for six CF human colon organoid lines, whereby representative CFTR genotypes are tested for basal CFTR function and response to CFTR-modulating drugs.

For complete details on the use and execution of this protocol, please refer to Dekkers et al (2016) and Berkers et al (2019).

GRAPHICAL ABSTRACT



Before you begin:

CRITICAL: All laboratory procedures related to colon organoid or cell cultures should be performed in a laminar flow to avoid contaminations and to protect the operator from biological risks.

A. Preparations

1. Prepare Dulbecco's Modified Eagles Medium with additional 1 % (v/v) pen- strep (10,000 U/mL) and 10 % (v/v) FBS (DMEM +/+) and pre=warm to 37° C

B. Wnt-3A conditioned medium (WCM) production

Note: *it is strongly advised to produce and mix multiple batches (2-3) of WCM to compensate for batch-batch variation during WCM production. Each round can produce around 1100 mL of WCM leading to a WCM pool of 2200 (mix of 2 batches)–3300 mL (mix of 3 batches) of which the total production time is between 20 (two batches) and 24 (3 batches) days.*

1. Thaw a cryovial with L-Wnt-3A cells by putting 13 mL of pre-warmed DMEM +/+ medium in a 15 mL tube (when thawing multiple cryovials, use a 50 mL tube with 30 mL DMEM +/+).
2. Transfer the cells from the cryovial to the 15 mL tube by resuspending the cells in DMEM +/+.
3. Centrifuge the 15 mL tube at 450 *g* for 5 min. Remove the supernatant and resuspend the cell pellet in 25 mL pre-warmed DMEM +/+ with additional 31.25 µL zeocin (final concentration 125 µg/mL).
4. Make sure that a homogenous cell suspension is obtained and transfer the cell suspension to a T175 flask.
5. Incubate the T175 flask for 3-4 days at 37 °C, 5% CO_2.
6. Split the cells when 90-100% confluency is achieved into 6 x T175 flasks:
7. Remove the DMEM +/+ from the T175 flask.
8. Gently wash the cells once with 5 mL PBS0 and discard the PBS0.
9. Add 1.5 mL 0.05 % Trypsin EDTA and leave at 18-23 ° C for 7 - 10 min until the cells are detached.
10. Add 10.5 mL DMEM +/+ medium, take up all the cells and resuspend until a homogenous cell suspension is achieved.
11. Add 2 mL DMEM +/+ of the cell suspension to each new T175 flask and top up to 25 - 30 mL of total DMEM +/+ per flask:
    > 5 x T175 flask in DMEM +/+
    > 1 x T175 flask in DMEM +/+ with zeocin, use this flask for the next production batch when the cells are 90-100% confluent again (usually after 3 - 4 days).

When the cells in the five T175 flasks containing DMEM +/+ are 90 -100% confluent (usually after 3 - 4 days), split the cells in 145 mm petri dishes:

12. Remove DMEM +/+ medium from the flasks and wash once with 5 mL PBS0.
13. Add 0.05 % Trypsin EDTA and leave at 18-23 ° C for 7 - 10 min.
14. Add 3.5 mL of DMEM +/+ per flask, pool the cells from all five T175 flasks, and resuspend until a homogenous cell suspension is achieved (total volume = 25 mL DMEM +/+ medium with cells).
15. Measure the cell concentration per mL and dilute the cell suspension needed for seeding in 50 145 mm tissue culture petridishes to create one (the first) batch with 1100 ml of WCM. For 50 145 mm (2 x 10^6 cells per 22 ml * 50=) 100 x 10^6 cells are needed in 1100 ml.
    - o Determine the cell concentration (with e.g. hemocytometer) [in #cells/ mL] = Y
    - o Calculate 100/ Y = X, the volume of the cell suspension to dilute in 1100 ml.
16. Make sure the cell suspension is homogeneous and add 22 mL of cell suspension per 145 mm tissue culture petridish.

<u>Note:</u> *When more than the necessary amount of cells is counted, a part of the cell suspension may remain unused or extra 145 mm petridishes can be seeded.*

17. Incubate the cells at 37 °C, 5 % CO_2 for 8 days (do not harvest the WCM/ DMEM +/+ before day 8)
18. Harvest the WCM from the petridishes in sterile 50 mL tubes and discard the petridishes with cells.
19. Centrifuge for 5 min at 650 *g* to remove floating cells and debris.
20. Filter medium through a 0.22 μm filter (stericups are preferable).
21. Store the WCM in 50 mL tubes or in 0.5 – 1 L containers at 4 °C.
22. Repeat the splitting of a 1 x T175 flask for the next batch as described above for 2-3 batches per WCM production.
23. WCM can be stored at 4°C and used for 2 months. Testing and approving of the WCM batches: WCM quality can be tested by culturing organoids with this specific WCM batch. Differentiated, non- budding round, thick-walled organoid structures indicate low Wnt activity (also see figure 10C). Running human colon organoid cultures often need time to adjust to a new batch of WCM. Judge organoid cultures after 2 weeks before making conclusions on the WCM quality.

<u>Note:</u> *A running L-wnt3a cell line can be used for the production of multiple batches of WCM until passage 18 - 20.*

C. Noggin conditioned medium (NCM) production

Note: HEK293T Noggin hFc cells are poorly adhesive cells (especially the first passage after thawing). At the first passage after thawing leave the cells for at least 5 days without touching. Make sure to handle the cells gently during washing with PBS0 to avoid detaching. An extra passaging step before starting the harvesting steps is strongly recommended to let the cells recover from the freeze-thawing step.

1. Thaw a cryovial with HEK293T Noggin hFc cells by putting 13 mL of pre-warmed DMEM +/+ in a 15 mL tube (when thawing multiple cryovials, use a 50 mL tube with 30 mL DMEM +/+).
2. Transfer the cells from the cryovial to the 15 mL tube by resuspending the cells with DMEM +/+.
3. Centrifuge the 15 mL tube at 450 *g* for 5 min. Remove the supernatant and resuspend the cell pellet in 40 mL pre-warmed DMEM +/+ with additional G418 (final concentration 500 µg/mL).
4. Make sure that a homogeneous cell suspension is achieved and transfer the cell suspension to a T175 flask.
5. Incubate the T175 flask for 7 days at 37 °C and 5% CO_2.
6. Split the cells when 90-100 % confluence is achieved into 6 x T175 flasks.
7. Gently remove the DMEM +/+ from the T175 flask.
8. Gently wash the cells once with 5 mL PBS0 and discard the PBS0.
9. Add 1.5 mL 0.05 % Trypsin EDTA, leave at 18-23 ° C for 7 - 10 min until the cells are detached.
10. Add 10.5 mL DMEM +/+ medium, take up all the cells and resuspend until a homogenous cell suspension is achieved.
11. Transfer the cell suspension to 15 mL tube
12. Count the cells (with e.g. hemocytometer)
13. Add $5*10^6$ cells in 25 mL culture medium and transfer to a new T175 flask:
    - 1 x T175 in culture medium with G418 (500 µg/mL), use this flask for the next batch (usually cells are confluent after 3 - 4 days).
    - X (depending on amount of cells) x T175 in culture medium without G418.

When the cells seeded in DMEM +/+ without G418 are ~90% confluent (usually after 3 - 4 days):

14. Remove the DMEM +/+ from the T175 flasks
15. Gently (to avoid detachment of the cells) add 50 mL of Ad-DF+++ per flask.
16. Incubate the flasks at 37 °C and 5% CO_2 for 8-10 days.

4

17. Harvest the Ad-DF+++ (NCM) on day 8 -10. Never harvest NCM before day 8 or when cells are not at least 100 % confluent.
18. Pool the NCM from the T175 flasks in sterile 50 mL tubes and discard the T175 flasks.
19. Centrifuge the NCM for 5 min at 650 *g* to remove floating cells and debris.
20. Filter medium through a 0.22 µm filter (stericups are preferable).
21. Store the NCM in 50 mL tubes or in 0.5 – 1 L containers at -20 °C .

Note: A running HEK293T-Noggin-hFc cell line can be used for the production of multiple batches of NCM until passage 18 - 20.

Note: No properly validated NCM quality/ quantity assay has yet been developed. After testing the functionality (on human colon organoid cultures) the NCM batches can be thawed, pooled and refrozen in aliquots and are stable at -20 ºC for at least one year. Try to avoid unnecessary freeze-thaw cycles.

Note: Different NCM batches can show much variation in Noggin activit. It is advisable to produce multiple batches and mix an NCM pool to ensure a minimal efficiency level of active NCM.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides and Recombinant Proteins		
0.05% Trypsin EDTA	Thermo Fisher Scientific : Invitrogen	#25300-054
Advanced Dulbecco's Modified Eagles Medium with Nutrient Mixture F-12 Hams (Ad-DF) 500 mL	Thermo Fisher Scientific: Invitrogen	#12634
B27 supplement	Thermo Fisher Scientific: Invitrogen	#17504-044
Calcein, AM	Life Technologies: Gibco	#C3100MP
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	#276855-1L
Dulbecco's modified Eagle medium (DMEM)	Thermo Fisher Scientific: Invitrogen	#31966-021
Fetal bovine serum (FBS, brand varies upon optimal batch selection)	Sigma/ Bovogen	
Forskolin	Sigma	#F3917-10mg
Gastrin	Sigma Aldrich	#G9145
Gentamicin	Life Technologies: Gibco	#15710-049
Glutamax	Thermo Fisher Scientific: Invitrogen	#35050
G418 (100 mg/mL)	Invivogen	#ant-gn-5
Human Epithelial Growth Factor (hEGF)	Peprotech	#AF-100-15
HEPES	Thermo Fisher Scientific: Invitrogen	#15630-056
Matrigel (important: protein concentration > 10 mg/mL)	Corning	#356231
N-Acetylcysteine	Sigma Aldrich	#A9165
Nicotinamide	Sigma Aldrich	#N0636
p38 MAPK inhibitor (p38i) (SB202190)	Sigma Aldrich	#S7067
Phosphate Buffered Saline 0 (without Ca and Mg)	Sigma/ Thermofisher/ sterile homemade	#D5652/ #14190250
Penicillin/ Streptomycin	Thermo Fisher Scientific: Invitrogen	#15140-122
Primocin (50 mg/ mL)	InvivoGen	#ant-pm-1
Recovery™ Cell Culture Freezing Medium	Thermo Fisher Scientific: Invitrogen	#12648010
Recombinant Human R-Spondin 3 Protein (hR-spondin-3)	R&D	#3500 – RS/ CF
TGFb type I Receptor inhibitor (A83-01)	Tocris	#2939
Ultrapure 0.5 M EDTA, pH 8	Thermofischer	#15575020
Vancomycin	Sigma Aldrich	#861987-250mg
VX-661	Selleckchem	#S7059
VX-770	Selleckchem	#S1144

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Y-27632 Dihydrochloride (ROCKi)	Abmole bioscience	#Y-27632
Zeocin (100 mg/mL) 1g	Thermo Fisher Scientific: Invitrogen	#R25001
Experimental Models: Cell Lines		
HEK293T – Noggin hFc cell line	Optimal Noggin-hFc producing subclone obtainable through the HUB: http://hub4organoids.eu/	n/a
L- Wnt 3A producing cell line	ATCC Optimal Wnt-3A producing subclone obtainable through the HUB: http://hub4organoids.eu/	#CRL-2647
Human colon organoids genotype G542X / G542X	HUB: http://hub4organoids.eu/	#HUB-02-D2-121
Human colon organoids genotype F508del / R1162X	HUB: http://hub4organoids.eu/	#HUB-02-D2-038
Human colon organoids genotype F508del/ F508del	HUB: http://hub4organoids.eu/	#HUB-02-D2-341
Human colon organoids genotype F508del/ G551D	HUB: http://hub4organoids.eu/	#HUB-02-D2-043
Human colon organoids genotype F508del/S1251N	HUB: http://hub4organoids.eu/	#HUB-02-D2-103
Human colon organoids genotype F508del / R117H - 7T	HUB: http://hub4organoids.eu/	#HUB-02-D2-004
Software and Algorithms		
Cell profiler (open- source)	McQuin et al., 2018	https://cellprofiler.org/
Zen blue®	Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html
Graphpad Prism	Graphpad	https://www.graphpad.com/scientific-software/prism/
Microsoft Excel	Microsoft Corporation	https://office.microsoft.com/excel
Other		
15 and 50 mL conical tubes	No recommended vendor	n/a
Microfuge tubes	No recommended vendor	n/a

REAGENT or RESOURCE	SOURCE	IDENTIFIER
100 mL (plastic) bottles	No recommended vendor	n/a
0.22 µm filters	No recommended vendor	n/a
Serological pipets	No recommended vendor	n/a
Micropipet filter tips + Micropipet tips without filter (200 µL)	No recommended vendor	n/a
Cryovials	No recommended vendor	n/a
24-wells flat bottom tissue culture plates / 96-wells flat bottom tissue culture plates	No recommended vendor	n/a
T175 cell culture flasks	No recommended vendor	n/a
145 mm petridishes	No recommended vendor	n/a
Cell Freezing Container (e.g. Mr Frosty)	No recommended vendor	n/a
Light/optical microscope appropriate for cell culture with standard ocular (10x) and 4x and 10x objective	No recommended vendor	n/a
Live cell imaging confocal microscope with 5x objective, incorporated incubator (allowing controlling temperature and CO2) and 488 nm laser	Recommended but not essential: Zeiss LSM800	n/a
Liquid nitrogen tank	No recommended vendor	n/a

Note: clinical reagents and materials mentioned in 'obtaining human (colon) rectal biopsies' (e.g. rectal suction biopsy device, forceps device, video endoscope) are not included in this list and are often hospital-specific. For information please contact your local gastroenterologist.

MATERIALS AND EQUIPMENT:

Culture Medium	Abbreviation	Description	Application
DMEM +/+	DMEM +/+	Dulbecco's Modified Eagles Medium with additional 1 % (v/v) pen- strep (10,000 U/mL) and 10 % (v/v) FBS	Preparation of conditioned media
Ad-DF+++	Ad-DF+++	Advanced Dulbecco's Modified Eagles Medium with Nutrient Mixture F-12 Hams (Ad-DF 500 mL + 1% (v/v) glutamax, 1% (v/v) hepes buffer and 1% (v/v) pen-strep (see table 3 for exact concentrations)	Preparation of conditioned media, handling of colon organoid cultures.
Human colon organoid medium -/-	2x CM -/-	Colon organoid culture medium with**out** WCM & R-spo3 (see stock preparation of human colon organoid medium components)	Preparation of 50 mL stock 2x human colon organoid medium saved at -20°C
Human colon organoid medium +/+	CM +/+	Colon organoid culture medium **with** WCM & R-spo3 (see stock preparation of human colon organoid medium components)	Human colon organoid medium used for regular culture and FIS
Noggin conditioned medium	NCM	Self-produced Noggin conditioned medium	10% of human colon medium
Wnt-3A conditioned medium	WCM	Self-produced Wnt-3a conditioned medium	50% of human colon medium +/+

Stock preparation of human colon organoid medium components
Timing ~3 h

Human colon organoid medium consists of several chemical components as well as self-produced conditioned media, such as Wnt-3A conditioned media (WCM) and Noggin conditioned media (NCM). In this protocol hR-spondin-3 (R-spo3) was preferred over self-produced R-spondin-1 conditioned media. However, a R-spondin-1 producing cell line is available and could be preferred due to cost reduction of media.

Table 1: overview of the stock preparation of the individual medium components with all relevant ordering and dissolvent information:

Compound	Amount	Concen-tration stock	End concen-tration colon medium +/+	Storage
Ad-DF+++	Add 5 mL Glutamax (100 x)		40%	8-12 weeks at 4 °C
	Add 5 mL HEPES (1M)			
	Add 5 mL Pen/Strep (10,000 U/mL / 100 x)			
B27	10 mL ◊direct use from bottle	-	2%	1 yr at -20 °C
N-acetylcysteine	815 mg Nac in 10 mL H_20	500 mM	1.25 mM	1 yr at -20 °C
hEGF	Add 2 mL filter sterilized PBS0-BSA (0,1 %) to 1 vial hEGF	0.5 mg/mL 10.000 x	50 ng/mL	1 yr at -20 °C
Nicotinamide	6 gr nicotinamide in 50 mL PBS0	1 M 100 x	10 mM	4 months at -20 °C
A83-01	10 mg A83-01 in 5 mL DMSO	5 mM 10.000 x	500 nM	3 months at -20 °C
P38 inhibitor (SB202190)	Add 500 µL DMSO to one bottle of 5 mg P38-inhibitor	30 mM 3000 x		3 months at -20 °C
Gastrin	Mix 0.5 mg gastrin to 2.383 mL PBS0	100 µM	5 nM	3 months at -20 °C
R-spo3	Reconstitute in PBS0	Varies per batch	250 ng/ mL	3 months at -80 °C
WCM	Collect from producing cell-line (see 2.1)	2 x	50%	2 months at 4 °C
NCM	Collect from producing cell-=line (see 2.2)	10 x	10%	1 yr at 20 °C

Preparing 2x colon organoid medium -/-

Timing ~1 h

Table 2 shows how to prepare 2x colon organoid medium (2x CM) -/- by addition of all reagents except WCM and R-spo3. It is advisable to calculate the medium amounts that will be used within the following 2.5 months and adjust the volume of 2x CM -/- preparation.

Table 2: Preparation of 2x colon organoid medium (CM) -/-.

Reagent	*Final conc.*	*100 mL total medium (50 mL 2x CM -/-)*
Ad-DF+++	36.8 %	36.8 mL
NCM	10 %	10 mL
B27	2 %	2 mL
N-acetylcysteine	1.25 mM	250 μL
hEGF	50 ng/mL	10 μL
Nicotinamide	10 mM	1 mL
A83-01	500 nM	10 μL
P38 inhibitor	10 μM	33 μL
Gastrin	100 μM	5 μL
Primocin	100 μg/mL	0.2 mL

Store 2x CM -/- in aliquots at – 20 °C for a maximum of 2.5 months. For example, aliquot 50 mL in 100 mL bottles so WCM can be added when preparing CM +/+ as described in the following section.

Human colon organoid medium +/+ preparation

Timing ~1 h

When preparing fresh CM +/+ for direct use in human organoid cultures, thaw a bottle of frozen 2x CM -/- over 8-12 hours at 4 °C (preferably in the fridge) and add WCM and R-spo3 the following day. Table 3 shows the ratio of adding WCM and R-spo3 to create 100 mL total volume of CM +/+. CM +/+ can be used for 10 days if stored at 4 °C.

Table 3. Preparation of colon organoid medium (CM) +/+

Reagent	**Final conc.**	**100 mL CM +/+**	**Storage**
2x CM -/ -	50 %	50 mL	10 days at 4 °C
WCM	50 %	50mL	2 months at 4 °C
R-spo3	250 ng/mL	Depends on LOT#	7 days at 4 °C

Step by Step Method Details:

Obtaining human (colon) rectal biopsies

Timing ~2 h

NOTE: both the forceps biopsy (A) and the rectal suction biopsy (B) are medical procedures which should only be performed by trained medical staff in a medical clinic. Make sure all necessary precautions are in place for a safe procedure.

CRITICAL: Clinical procedures to isolate rectal or colon biopsies from patients should ALWAYS be performed by trained medical staff in a medical clinic. Make sure all necessary precautions are in place for a safe procedure.

1. Fill a 50 mL conical tube with +/- 20 mL of PBS0 at 4 °C
2. If biopsies are transported over long distance or long duration (>8 h) before crypt isolation procedure can take place, biopsies should be stored at 4 °C in Ad-DF +++ with additional gentamicin (50 µg/mL) and vancomycin (50 µg/mL).

CAUTION: Authorized personnel should wear gloves and a physician's coat. This is the minimum protection that should be worn when working in the OR (operating room). Make sure all the apparatus are calibrated and sterilized.

CRITICAL: *Procedures should not take place:*

If the patient suffers from thrombocytopenia (less than 50×10^9 thrombocytes /L).

If the patient suffers from coagulation disorder.

If the patient is currently suffering from severe inflammation of the intestine.

In case of visibility problems during the endoscopy.

Possible complications during and after the procedure

Perforation of the intestinal wall.

Severe or persistent bleeding.

Attention points

The nurse notifies the physician if the biopsies are of excellent, decent or inferior quality (see figure 3).

Be considerate with regards to the position and signs of any pain or discomfort of the patient.

A. Forceps biopsy procedure:

Additional information on forceps biopsy procedure including video can be found elsewhere (Servidoni et al., 2013).

Note: A nurse/endoscopy-assistant guides the patient through the process and assists the physician throughout the procedure.

CAUTION: The (jumbo) forceps should be closed and pulled back when entering or exiting the endoscopic instrumentation channel.

CAUTION: The procedure should not take place in case there are visibility problems during the endoscopy.

Note: Before starting the procedure, be sure you have read and understood all the instructions detailed in this paragraph.

1. Make sure the patient agrees to the procedure before starting and the appropriate informed consent form is signed.
2. The nurse opens the 50 mL collection tube containing PBS0 or storage media (Ad+++ with additional gentamicin (50 µg/mL) and vancomycin (50 µg/mL)) at 4°C and properly labelled for the rectal biopsies (with clean gloves).
3. The nurse proceeds by opening a new sterile jumbo forceps packaging, removing the cover at the end of the forceps around the mouth of it and presents it to the physician.
4. The physician enters the forceps inside the endoscopic instrumentation channel.
5. The nurse/assistant opens or closes the forceps as instructed by the physician.
6. When removing the forceps out of the endoscopic instrumentation channel it should be cleaned with a gauze to avoid blood spatters. The biopsies are collected in the collection tube.

Note: If the rectal/ colon biopsy is sticking to the jumbo forceps, use a sterile needle or sterile tweezers to remove the biopsy.

7. Rinse the forceps in a cup of clean water before re-entering the jumbo forceps into the endoscope to collect more biopsies from the patient. **Make sure the forceps is clean.**

8. Repeat steps 4-7 until a minimum of 4 good quality biopsies have been successfully isolated and collected (all biopsies can be collected in the same tube).
9. After the procedure the nurse firmly closes the collection tube and takes off her gloves.
10. If there is more than one biopsy collection tube, make sure it is marked and numbered by the nurses.
11. Maintain the collection tube with the biopsies on ice at ~4°C in a styrofoam box.
12. After the procedure is finished the patient should remain in the hospital for an additional hour to check for potential rectal bleeding. In case of severe or persistent rectal bleeding a qualified physician should be consulted.
13. When no complications are observed the patient can be discharged.
14. Inform the patient about potential late onset rectal bleeding. In case of late onset rectal bleeding a qualified physician should be consulted.

B. Obtaining biopsies by rectal suction device

A nurse/endoscopy-assistant guides the patient through the process and assists the physician throughout the procedure.

<u>Note:</u> Before starting the procedure, make sure to have read and understood all the instructions detailed in this paragraph

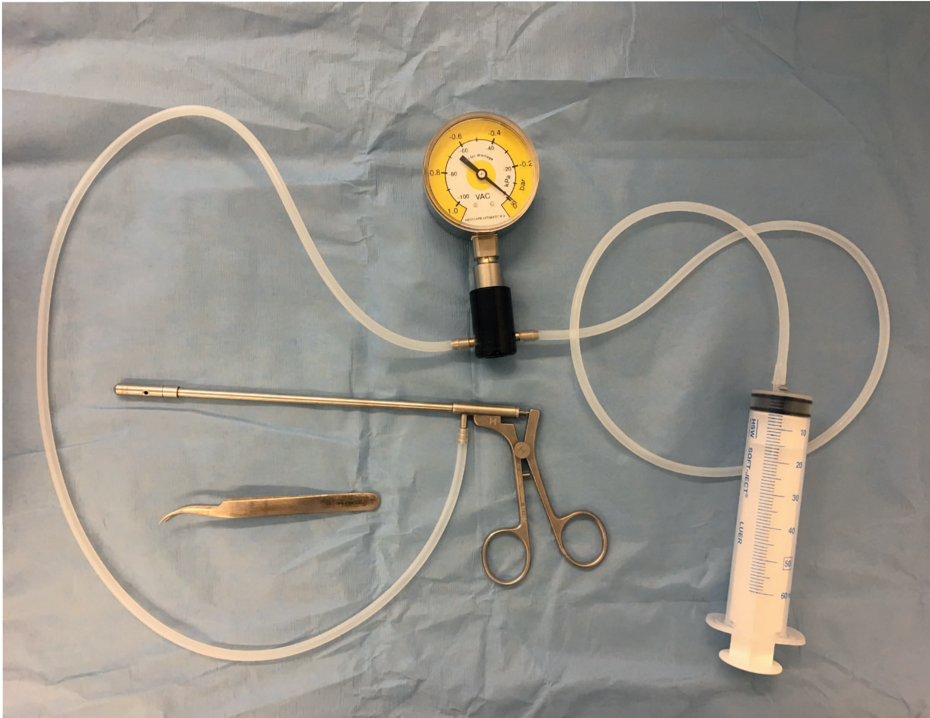


Figure 1. Rectal suction biopsy device with manometer for defined suction pressure and syringe to create suction.

1. Make sure the patient agrees to the procedure before starting and the appropriate informed consent form is signed.
2. Ensure an appropriate bowel preparation: apply an enema to the patient (Sodium phosphate enema (2.5 mL* (per) kg body weight; maximum dose 130 mL) by inserting the sodium phosphate solution rectally +/- 2-3 h before the biopsy procedure.
3. Wait for the result of the enema.
4. Test the rectal suction device for vacuum pressure before using it in the patient (see figure 1).

5. The nurse opens the 50 mL collection tube containing PBS0 or storage media (Ad-DF+++ with additional gentamicin (50 µg/mL) and vancomycin (50 µg/mL)) at 4°C and properly labelled for the rectal biopsies (with clean gloves).
6. Apply lubricant (e.g. K-Y Jelly) to the rectal suction biopsy device.
7. Carefully introduce the suction biopsy device rectally, approximately 5 cm distance from the anal verge.
8. Position the opening of the suction biopsy device between 45 and 90 degrees laterally from the dorsal side to avoid arteries (see figure 2).
9. Obtain a biopsy from the rectum/ colon, with a defined suction pressure of 30 kPa/0.3 Bar.
10. Release the rectal (colon) biopsy from the device by twirling and shaking the frontal side in the collection tube containing 20 mL storage media at 4 °C (either PBS0 or Ad-DF+++ with additional gentamicin (50 µg/mL) and vancomycin (50 µg/mL)) and until the biopsy is visible in the fluid.
11. Repeat steps 5-10 until a minimum of 4 good quality biopsies have been successfully isolated and collected (all biopsies can be collected in the same tube).
12. After the procedure the nurse firmLy closes the collection tube and takes off her gloves.
13. Maintain the collection tube with the biopsies on ice at ~4°C in a styrofoam box
14. After the procedure is finished the patient should remain in the hospital for an additional hour to check for potential rectal bleeding. In case of severe or persistent rectal bleeding a qualified physician should be consulted.
15. When no complications are observed the patient can be discharged.
16. Inform the patient about potential late onset rectal bleeding. In case of late onset rectal bleeding a qualified physician should be consulted.

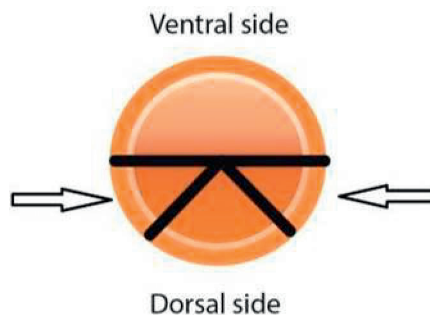


Figure 2. Cross-sectional view of the colon with advised positioning of the rectal suction biopsy device opening

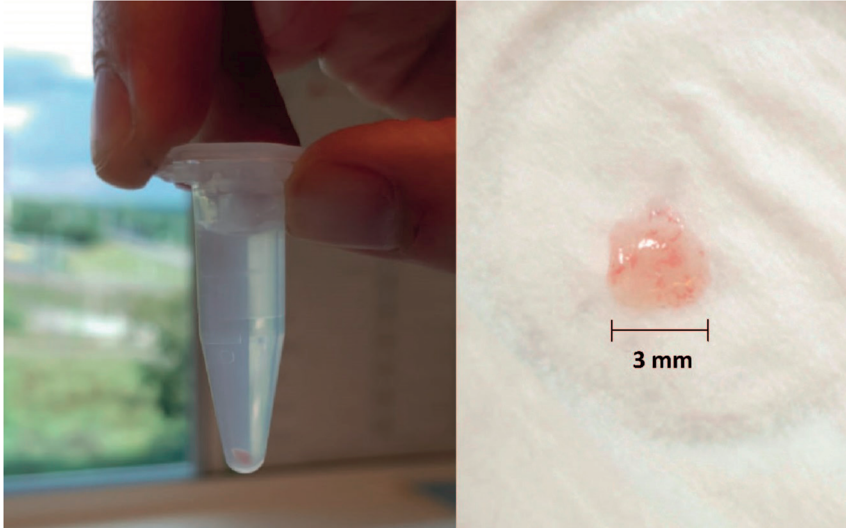


Figure 3 . Example of a goo d quality biopsy. Note the pink colour and round shape.

Transport of biopsies to the laboratory

CRITICAL: Intestinal crypts can be retrieved from a rectal/ colon biopsy (hereafter referred to as “colon biopsy”) with a high efficiency (> 95% success rate) within 48 h of the biopsy collection. However, tissue quality gradually decreases over time leading to a reduced chance of successfully isolating crypts from the biopsies. Therefore, immediate transport of the colon biopsies at 4 °C in AD-DF+++ is essential to ensure successful crypt isolation and establish efficient colon organoid cultures.

Note: Good quality colon biopsies should be round and have a slightly pink to red colour (see figure 3 & 4). When you are uncertain about the quality of the rectal biopsies, please check if intestinal crypts are visible under the light microscope. Good quality biopsies show a honeycomb- like structure in which the stem cell crypts reside. If this structure is not visible, the biopsy is of bad quality (figure 4B).

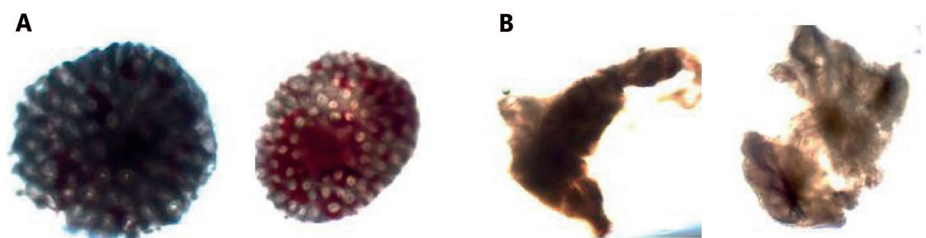


Figure 4. Examples of biopsies as viewed under a microscope (bright field, 4 x magnification). (A) Good quality biopsies, note the honeycomb structure indicative of intestinal crypts. (B) Bad quality biopsies, this can be due to lack of tissue depth of the biopsy leading to an absence of intestinal crypts.

1. The colon biopsies should be stored on ice or at 4°C (fridge) in properly labelled 50 mL polypropylene conical tubes containing 30 mL of PBS0. Make sure the tissue is NOT frozen.

Note: If the biopsies are transported over long distances or over a long duration (>8 hs) before the crypt isolation procedure can be initiated, the biopsies should be stored in Ad-DF+++ with additional gentamicin (50 μg/mL) and vancomycin (50 μg/mL) at 4°C.

2. Make sure the 50 mL conical tube containing the colon biopsy in the storage medium is tightly closed and properly labelled. The date of the biopsy collection should be written on the tube (preferably on a sticker).
3. During the biopsy transport, the temperature needs to be 4°C. Please use cold gel or ice packs during the shipment.

CRITICAL: *Wet ice should not be used for the shipment, since thawing ice might damage the labelling of the tubes.*

Organoid crypt isolation from human colon (rectal) biopsies
Timing ~3 h

CRITICAL: All laboratory procedures related to colon organoid or cell cultures should be performed in a laminar flow to avoid contaminations and to protect the operator from biological risks.

CRITICAL: Always keep matrigel at 4°C or on ice to prevent polymerization above 10 °C.

CRITICAL: Pre-warm 24- and 96-wells plates used for organoid culturing to 37 °C for a minimum of >1 day. This is essential to ensure that after the colon organoids are plated, the matrigel polymerizes in the well efficiently and forms stable drops.

A. Preparations

1. Prepare cold Ad-DF+++ and cold PBS0 (4 °C).
2. Thaw (and keep) matrigel on ice or at 4 °C and dilute 1:1 with cold CM +/+ (= 50 % matrigel).
3. Prepare CM +/+ with additional gentamicin (50 μg/mL) and vancomycin (50 μg/mL) to reduce the risk of infection.

B. EDTA incubation

CRITICAL: In every step of this procedure, when handling the biopsies with pipettes, it is necessary to pre-coat the pipettes with Ad-DF+++ by aspirating media into the pipettes and discard the media. This covers the inside of the pipets with liquid and prevents biopsies from adhering to the plastic.

1. Wash the colon biopsies several times in cold PBS0 until the supernatant is clear using the following procedure:
   a. Pipet the biopsies up and down ~10 times in 10 mL with a 10 or 25 mL pipet.
   b. Let the biopsies settle at the bottom for 30 seconds.
   c. Remove the supernatant and add 10 mL of clean cold PBS0.
   d. Repeat these steps 2-4 times until the biopsies and supernatant are clear of debris.
2. Discard the supernatant and add 10 mL of clean PBS0 to the biopsies and supplement it with 200 μL ultrapure EDTA pH 8, 0.5 M (final concentration 10 mM).

3. Place the tube on a roller mixer for 90 - 120 min at 4 °C. The incubation time is dependent on the time between the biopsy collection and the start of the crypt isolation procedure. The longer the interval between biopsy collection and isolation procedures, the longer it is required to incubate. Crypts can be isolated from colon biopsies up until 48 h after biopsy collection but crypt yield decreases over time.

C. Isolate intestinal crypts from biopsies

1. Allow the colon biopsies to settle at the bottom of the tube.
2. Discard the supernatant (PBS0 + EDTA).
3. Add 3 mL cold PBS0 to the tube containing the biopsies and pipet the biopsies up and down vigorously 10 - 20 times. Crypts are released from the biopsies and float in the PBS0, which is visible by eye through the observation of the solution becoming more cloudy. If no crypts emerge from the colon biopsies after 90-120 min of EDTA incubation add new PBS0 + EDTA and incubate for another 60 min.
4. Allow the biopsies to settle at the bottom and transfer the supernatant with the crypts to a clean 15 mL tube.
5. Add fresh 3 mL of cold PBS0 to the biopsies again and repeat previous steps until no more crypts detach or until a sufficient amount of crypts are transferred to the clean tube.
6. Add Ad-DF+++ to top up the solution containing the crypts in the 15 mL tube and centrifuge the crypts at 130 g for 5 min at 4 °C.
7. Gently remove the supernatant, the crypt cell pellet is vulnerable and can easily be lost by aspiration.
8. Add 10 mL of Ad-DF+++ to the crypt pellet for a second washing step and centrifuge at 130 *g* for 5 min at 4 ºC.

D. Plating intestinal crypts in matrigel

1. For the first passage (p.0) make sure to use a separate 24-wells plate per patient sample since isolated crypts from primary intestinal material during the first passage are most susceptible to infections.
2. Keep the intestinal crypt pellet on ice.
3. Analyze the size of the pellet by eye to determine the appropriate volume of 50% matrigel.
    - o Usually, 4 - 6 wells per biopsy is sufficient for p.0. However, a higher number of wells can be used if the crypt yield is very high (7 – 12 wells) or a lower number when the yield is low (1-3 wells).
4. To ensure the crypt density is not too low, first resuspend the crypt pellet in 100 ml 50 % matrigel.

5. Check the crypt density by plating a test droplet and view under the light microscope. Preferably do not plate out crypts too dense to allow for efficient (out)growth and proliferation of the structures. The ideal density is 15- 20 crypts per 7.5 µL of matrigel drop.

Note: the crypt density can be adjusted according to the observations in the test droplet.

Too dense: If crypts are too densely seeded, dilute the sample in more matrigel and seed additional wells with the extra matrigel.

Too sparse: If crypts are too sparsely seeded, crypts can be centrifuged at 130 g 5 min at 4 °C and resuspended in smaller volumes of matrigel to increase the density.

Note: Prevent bubble formation during pipetting and plating.

6. Plate 4* 7.5 µL of matrigel with about 15-20 crypts per drop of a (pre-warmed) 24-wells plate.
7. Resuspend the matrigel after plating out 3 wells to ensure the crypts have not settled at the bottom of the microcentrifuge tube.
8. Incubate 30 min. at 37 °C.
9. Add 500 µL of pre-warmed CM +/+ with additional gentamicin (50 µg/mL) and vancomycin (50 µg/mL). An example of crypts directly after isolation can be found in Figure 5.

Note: gentamicin and vancomycin are only added to CM +/+ in the first week of culturing

10. Incubate for 7 days at 37 °C, 5 % CO_2.

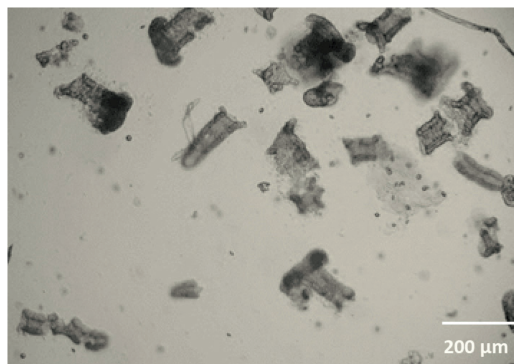


Figure 5. Crypts on day 0 directly after isolation (bright field, 4 x magnification).

E. First week of culture

Note: Crypts close quickly and start to proliferate (visible as "budding") after several days (see Figure 6).

1. Often check wells for infections. When any of the wells shows clear signs of infection, aspirate the medium from the well and add 1 mL ethanol for 30 s. Aspirate ethanol and matrigel drops and re-add 1 mL of ethanol for 30 s. Aspirate and leave well empty (important: leaving ethanol creates ethanol vapour in the plate, which leads to cell death in the other wells). This approach prevents cross contamination of other wells from this patient sample.
2. Refresh the wells with 500 µL colon organoid medium +/+ with additional gentamicin (50 µg/mL) and vancomycin (50 µg/mL) every 2 - 3 days (e.g. Monday, Wednesday, Friday). If crypts are isolated on a Friday, add 1 mL CM +/+ instead of 500 µL (with additional gentamicin and vancomycin) to ensure optimal growth during the weekend

Note: To remove the media, use a new tip for every well to decrease infection and well to well contamination risks.

3. Once the crypt structures are budding, closed and proliferated (see figure 6), passage them as described in 5.1. This can usually be performed around day 6 - 8.

Note: FIS experiments should only be performed after a minimum of two passages after isolation.

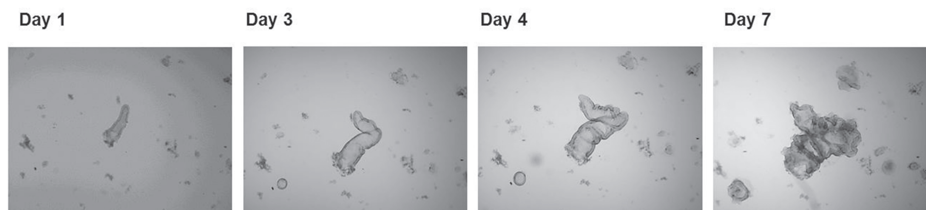


Figure 6. Development of crypt into budding organoid structure on day 1; 3; 4 and 7 (passage 0). Arrows point to proliferating crypt and subsequent organoid structure (bright field, 4 x magnification).

General handling and passaging of human colon organoid cultures
Timing ~3 h

CRITICAL: Pre-warm 24- and 96-wells plates used for organoid culturing to 37 °C for a minimum of >1 day. This is essential to ensure that after the colon organoids are plated, the matrigel polymerizes in the well efficiently and forms stable drops.

A. Preparations

1. Prepare cold Ad-DF+++ and warm CM +/+.
2. Thaw (and keep) matrigel on ice or at 4 ºC and dilute 1:1 with cold CM +/+ (= 50 % matrigel).

Note: the splitting ratio should be usually 1:4 - 1:8 24-wells. If seven day old organoid cultures cannot be split according to this ratio because they have not proliferated sufficiently, check the medium quality or reseed organoids in 1:1 wells without disruption to allow more time to recover or proliferate until disruption.

B. Passaging colon organoids

1. Discard the CM +/+ from the wells containing the colon organoids.
2. Detach the matrigel with the organoids by aspirating 1 mL Ad-DF+++ and forcefully dispensing the medium directly onto the matrigel drops in the well several times with a p1000 filter tip. If necessary gently scrape the well with the end of the pipet tip to detach residue.
3. Transfer the colon organoid suspension to a sterile 15 mL tube on ice. Several wells (up to 12) can be combined in the 1 mL.
4. Put a p200 tip (without filter) on top of a p1000 filter tip (see figure 7) and place the tip in the 1 mL organoid suspension on the bottom of the tube. Mechanically shear/disrupt the organoid structures by pipetting the 1 mL organoid suspension up and down 15-20 times through the p1000/ 200 tip combination. The organoids will be broken up into smaller parts. Resuspend with firm speed and fully take up and dispense the 1 mL suspension. Slow, mechanic shearing does not create efficient disruption. Minor foam formation is normal but try to prevent it by avoiding foam aspiration or dispension.

Figure 7. Put a p200 tip on top of a p1000 filter tip to disrupt the organoid structures.

Note: steps 5 and 6 are relevant when colon organoid cultures are passaged before performing a FIS experiment or to clean an organoid culture that has many differentiated structures which need to be removed (see figure 10C). Otherwise proceed with step 7.

5. Add 5 mL of cold Ad-DF+++ to the 1 mL of disrupted organoids.
6. Clean up the organoid suspension by resuspending the organoids and let the bigger, differentiated organoid structures sink down in a tube angles at 70° for 10 s (see figure 8). Take up the smaller organoids by removing 1 mL from the top of the medium and transfer this to a sterile 15 mL tube on ice. Repeat this step until only 0.5 - 1 mL medium with bigger structures is left.

Note: if many large structures are still left in the remaining 1 mL after steps 5 and 6 (visual by eye), repeat steps 4-6 but during step 4 only disrupt 10 times.

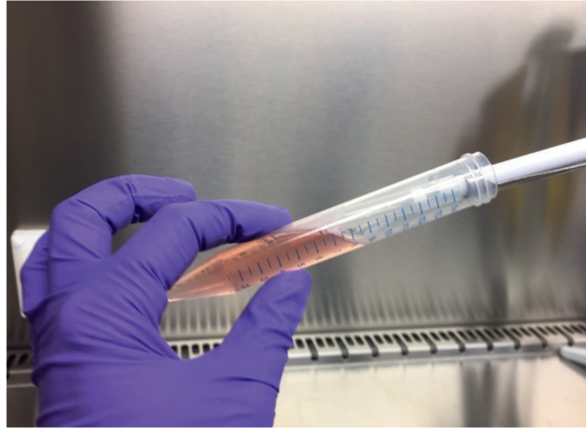


Figure 8. Hold the 15 mL tube tilted at 70 ° and transfer the organoids from the top of the medium to a new tube.

7. Top up the 15 mL tube containing the smaller, disrupted organoids with 10 mL cold Ad-DF+++.
8. Centrifuge at 130 *g* for 5 min at 4 °C.
9. Aspirate the supernatant and keep the organoid pellet.

CRITICAL: the colon organoid pellet is vulnerable and can be easily lost through aspiration of the supernatant. Be careful while aspirating the media: it is best to leave > 50 µL of supernatant on the organoids to avoid pellet aspiration. Remove the last supernatant with a p200 tip.

C. Plating colon organoids in 24-well plates

1. Resuspend the organoid pellet in an appropriate volume of 50% matrigel. The volume of matrigel needed is 30 µL per seeded well of a 24-wells plate assuming enough organoids have been passaged.

Example: *2 wells of a 7-day old, good quality organoid culture can be split into approximately 8 - 16 new wells of a 24-wells plate. To avoid too sparsely seeded wells, it is advisable to first resuspend in a matrigel volume sufficient to seed 6 wells. Therefore, the pellet can be resuspended in approximately 180 µL of 50% matrigel. In case the organoids are too densely seeded, the organoid suspension can be diluted by adding more matrigel.*

2. Check the number of organoids in a 10 ml test drop of matrigel by light microscopy, to decide if the organoid suspension should be further diluted (the

density should be 15-30 structures per drop, see figure 9B). Always recheck a new test drop after further dilution.

3. While preventing bubbles, plate 4* 7.5 μL matrigel with a p20 tip per well in a (pre-warmed) 24-well plate (see Figure 9). Slightly tilting the plate helps to deposit the drops in the desired positions.

Note: Organoid structures will quickly settle at the bottom of the tube within 30 seconds so frequently resuspend the organoid suspension with a p200. While resuspending keep the tube on ice to prevent the matrigel from solidifying in the tube.

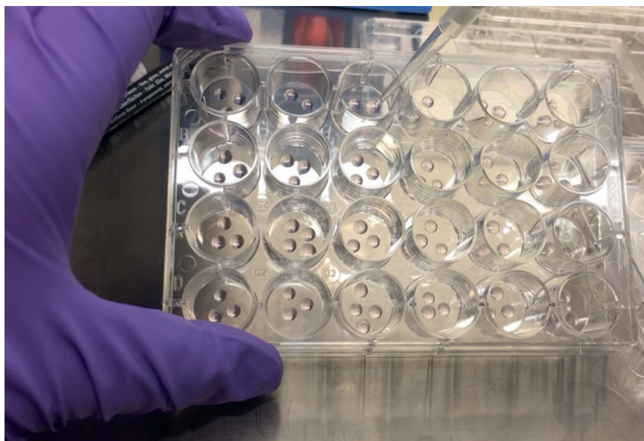


Figure 9. Add 4 drops of 7.5 μL containing organoids in a pre-warmed 24-wells plate. Slightly tilting the plate helps to deposit the drops in the designated location.

4. Incubate the plate for 15 - 20 min at 37 °C.
5. Add 500 μL of pre-warmed of CM +/+ per well.
6. Incubate the organoid cultures for 7 days at 37 °C, CO_2 5% and refresh the wells with 500 μL of CM +/ + per well, every 2 - 3 days (Monday, Wednesday, Friday).

Note: Human colon organoids can usually be passaged every 7 days. Therefore, it is important to regularly check the quality of the organoid cultures. Good quality organoid cultures show a stem cell phenotype which is characterized by efficient budding structures. For wild type (or very mild CF genotype) colon organoids a clear pre-swollen lumen can be seen with bright field imaging compared to colon organoids with a CF genotype where no lumen is visible (see Figure 10A and B).When colon organoids lose their stem cell phenotype (usually due to insufficient Wnt-3A activity in the WCM) the organoids appear as thick-walled, differentiated structures (see Figure 10C). These bad quality organoid cultures are not appropriate for CFTR- related experiments since this leads to unreliable results.

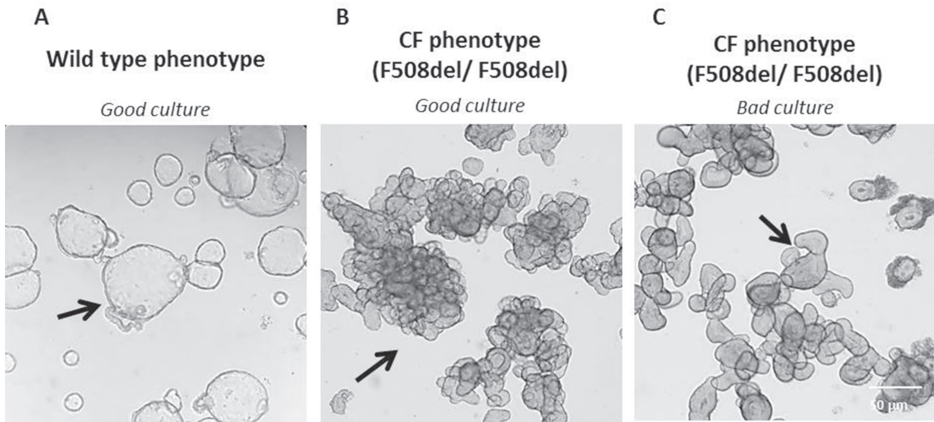


Figure 10. Examples of 7 day old human colon organoid cultures (bright field, 4 x magnification). A. Typical pre-swollen stem cell phenotype of a high quality, proliferated wild type (non-CF) human colon organoid culture. B. Non-swollen stem cell phenotype of a high quality, proliferated CF colon organoid culture. C. Example of a bad quality CF colon organoid culture with thick-walled, differentiated structures with decreased stem cell phenotype which is often due to low activity of Wnt-3A in WCM.

Freezing human colon organoid cultures

Timing ~2 h

Two different methods of organoids freezing are described here:

A. Freezing trypsinized human colon organoids after 1-3 days replating (Freezing protocol A). This is an efficient method to store source colon organoid samples in a master cell bank.
B. Freezing small organoid structures directly after disruption (Freezing protocol B); This is a fast, timesaving and efficient procedure appropriate for storing a local working cell bank.

1. Disrupt and freeze organoids when well is full (7 - 10 days cultured organoids)
2. Freeze one full well (of a 24-wells plate) per cryovial which should be sufficient to plate into 3-4 new wells after thawing.
3. Prepare cold Recovery™ Cell Culture Freezing Medium.
4. Prepare cold Ad-DF+++
5. Prepare ROCKi 10 mM stock.
6. Prepare warm CM +/+ with additional ROCKi 10 μM.
7. For freezing protocol A: thaw (and keep) matrigel on ice and dilute 1:1 with cold CM +/+ (= 50 % matrigel)
8. Prepare 0.05% Trypsin EDTA to 18-23 ° C

A. Freezing protocol A

1. Perform step 1-4 and 7,8 of ‘General handling and passaging of human colon organoid cultures’, part B.
2. Aspirate and discard the supernatant and add 4 mL of 0.05% Trypsin EDTA, and vortex for 30 s.
3. Put the tube in a warm water bath at 37 °C for 1 min and vortex vigorously for 30 s.
4. Inspect the solution in the tube by horizontally placing the tube on the light microscopy stage (4x objective), adjust focus so that organoids in solution are in visible. Observe the size of the organoid structures. If intact organoids are still visible put the tube in a warm water bath at 37 °C for an additional 1 min and vortex vigorously for 30 sec.
5. When the organoids are sufficiently disrupted (~100 x smaller), add 8 mL of Ad-DF+++ to neutralize the trypsin and resuspend 10 times the organoid suspension.
6. Centrifuge the tube for 3 min at 450 *g* at 4 °C.

7. Aspirate and discard the supernatant and add the required amount of medium and matrigel to the organoid pellet.

Note: For freezing, organoids must be seeded in a 1:1 ratio after trypsinization. Thus, 1 well of organoids from a 24-wells plate can be seeded into 1 new well of a 6-well tissue culture plate.

8. Mix the organoid suspension by resuspending without creating bubbles.
   - *Tip: Avoid bubbles by not fully aspirating and dispensing the pipet, but always keep a small amount of liquid in the tube.*
9. Seed 250 µL in a single well of a pre-warmed 6-well tissue culture plate by seeding 25 * 10 µL matrigel drops .
10. Place the plate in the incubator at 37 °C and leave the matrigel to solidify for 20-30 min.
11. Add 2.5 mL of fresh CM +/+ + ROCKi in each 6-well plate well and transfer the plate to the incubator.

Note: 1-2 day old organoid cultures are ready to be frozen. This increases the efficiency of survival after thawing.

12. Detach the matrigel and organoids by aspirating 1 mL Ad-DF+++ and forcefully dispensing the medium directly onto the matrigel drops in the well several times with a p1000. If necessary gently scrape well with end of pipet tip to detach everything.
13. Transfer the organoid suspension to a sterile 15 mL tube.
14. Wash the wells with another 1 mL of Ad-DF+++ and transfer to the same 15 mL tube.
15. Fill up the 15 mL tube with with 12 mL of cold Ad-DF+++ and pipet up and down with a 5 mL pipet.
16. Centrifuge the suspension for 3 min at 450 *g* at 4 °C, remove the supernatant and keep the pellet.
17. Dissolve the organoid pellet with cold Recovery™ Cell Culture Freezing Medium and pipet up and down to properly resuspend all the organoid structures.

Note: one full well from a 6-well plate is frozen in 1 mL of Recovery™ Cell Culture Freezing Medium and is divided over 2 cryovials. Each vial should contain enough cells to be thawed into ≥ 4 wells of 24-well plate.

18. Transfer 0.5 mL of organoids suspension in Recovery™ Cell Culture Freezing Medium to sterile cryovials.
19. Place the cryovials at -80°C in a cell container that will freeze the organoids 1°C per minute (e.g. mr Frosty).

20. After 24 h, transfer the cryovials for storage in liquid nitrogen.

B. Freezing protocol B

1. Perform step 1-4 and 7,8 of ‘General handling and passaging of human colon organoid cultures’, part B. However, shear/ disrupt the colon organoids 30 – 35 times to create even smaller organoid structures.
2. Add cold Recovery™ Cell Culture Freezing Medium
   Use 1 mL of Recovery™ Cell Culture Freezing Medium in 1 cryovial per isolated well. 1 frozen well in 1 cryovial can be thawed into 3- 4 wells of a 24 - well plate
   > pellet of 1 full well + 1 mL Recovery™ Cell Culture Freezing Medium or FM > add to 1 cryovial
   > pellet of 3 full wells + 3 mL Recovery™ Cell Culture Freezing Medium or FM > divide over 3 cryovials
3. Place the cryovials at -80°C in a cell container that will freeze the organoids 1°C per minute (e.g. mr Frosty).
4. After 24 h, transfer the cryovials for storage in liquid nitrogen.

Thawing human colon organoids from frozen nitrogen stock vials
Timing ~3 h

CRITICAL: Pre-warm 24- and 96-wells plates used for organoid culturing to 37 °C for a minimum of >1 day. This is essential to ensure that after the colon organoids are plated, the matrigel polymerizes in the well efficiently and forms stable drops.

Note: Human colon organoids cultures can be thawed from two different types of nitrogen stock vials: Thawing cultures that have been collected after 2-3 days in culture after trypsinization (freezing protocol A) or thawing cultures from vials with small organoid structures frozen directly after disruption (freezing protocol B).

1. Prepare warm Ad-DF+++ (37 °C).
2. Thaw (and keep) matrigel on ice or at 4 °C and dilute 1:1 with cold CM +/+ (= 50 % matrigel)
3. Prepare ROCKi 10 mM stock.
4. Prepare warm CM +/+ with additional ROCKi 10 μM.

<u>*Note:*</u> Addition of ROCKi is only necessary during the first week after thawing

A. Colon organoid thawing procedure

1. Add 13 mL pre-warmed Ad-DF+++ in a 15 mL tube
2. Rinse the outside of the cryovial with warm water or place in 37 °C water bath until the cell suspension in the vial is thawed. Clean the outside of the cryovial with 70 % EtOH. Alternative: first clean the outside of the cryovial with 70 % EtOH and then add warm Ad-DF+++ to the organoid suspension to thaw it.
3. Rapidly transfer organoids to 15 mL tube with 13 mL Ad-DF+++ (37 °C).

When thawing colon organoids frozen according to freezing protocol A:

1. Gently mix the suspension and centrifuge 450 *g* for 5 min at 4 °C.
2. Discard supernatant and resuspend pellet in 100 μL of 50 % matrigel.
3. Check the organoid density under the microscope by seeding a 7.5 μL matrigel test drop. The matrigel drop should contain >100 structures of very small colon organoid structures. Organoids frozen according to freezing protocol A should be thawed and seeded with higher density due to the smaller sized structures compared to protocol B. To ensure optimal outgrowth and budding of the colon organoid structures it is suggested to seed multiple densities to cover the most optimal density for recovery and proliferation.

4. Plate 4* 7.5 µL matrigel drops without bubbles to a single well of a (pre-warmed) 24-wells tissue culture plate. Usually one cryovial should contain enough organoids to seed in 3-4 wells.
5. Dilute the remaining colon organoid suspension 1:1 with matrigel and plate two additional wells.
6. Again, dilute the remaining colon organoid suspension 1:1 with matrigel and plate the fourth well.

When thawing colon organoids frozen according to freezing protocol B:

1. Gently mix the organoid suspension and centrifuge 130 *g* for 5 min at 4 °C.
2. Discard the supernatant and resuspend the organoid pellet in 100 µL of 50 % matrigel.
3. Check the organoid density under the microscope: seed the organoid structures with a 10-20% higher density compared to organoids already in culture, aiming for 50-70 structures per drop (10-20% of the structures are usually not viable). Organoids frozen according to freezing protocol B should be thawed and seeded with lower density due to the larger sized structures compared to protocol A.
4. Plate 4* 7.5 µL matrigel drops without bubbles per single well of a (pre-warmed) 24-wells tissue culture plate. Usually one cryovial should contain enough organoids to seed in 3-4 wells. Colon organoids will settle to the bottom quickly, so resuspend the organoids in the matrigel suspension frequently while plating.
5. Incubate a maximum of 10 min at 37 °C. Thawed human intestinal organoids are sensitive to being left without warm CM +/+
6. Add 500 µL of (pre-warmed) CM +/+ with additional ROCKi inhibitor (10 µM)to each well. Refresh the medium with 500 µL CM +/+, every 2 - 3 days (Monday, Wednesday, Friday).
7. Incubate at 37 °C, 5% CO_2 for 1 week.

<u>CRITICAL:</u> Monitor the progress of the thawed organoid structures daily.

Forskolin induced swelling (FIS) assay
Timing ~5 h

The FIS assay was developed to measure CFTR function in human colon organoids. Upon forskolin (fsk) stimulation (which indirectly activates CFTR through cyclic adenosine monophosphate (cAMP)), CFTR function can be assessed by observing the subsequent swelling of human colon organoids. Organoid swelling occurs through chloride excretion into the organoid lumen and osmosis. The assay can determine residual CFTR function and the effect of CFTR modulators.

CRITICAL: Pre-warm 24- and 96-wells plates used for organoid culturing to 37 °C for a minimum of >1 day. This is essential to ensure that after the colon organoids are plated, the matrigel polymerizes in the well efficiently and forms stable drops.

A. Preparations

1. Incubate 96-well plates at 37 °C > 7 days. It is important to have warm plates to prevent dislodging of matrigel drops.
2. Prepare cold Ad-DF+++.
3. Thaw (and keep) matrigel on ice and dilute 1:1 with cold CM +/+ (= 50 % matrigel).
4. Prepare warm CM +/ +.

B. Plating human colon organoids (day 1)

<u>Note:</u> *Some CFTR modulators (e.g. correctors) require longer periods incubation. Example: correctors VX-809 and VX-661 require 24 h incubation and are added directly to the CM +/+ on the day of the organoid plating, whereas potentiator (e.g. VX-770) should be added directly before the FIS measurement. Take this into account when CM +/+ is added. See* ‘European FIS validation & standardization protocol’ *for corrector preparations.*

1. Perform step 1-9 of ‘General handling and passaging of human colon organoid cultures’, part B.
2. Resuspend the organoid pellet in 50% matrigel (plating volume = 4 µL * amount of wells + 50 µL)
    - o With good quality organoid cultures, one full well of organoids from a 24-wells plate can be seeded into 10 - 20 wells of a 96-wells plate

Example: 8 wells of a 7-day, good quality organoid culture can be split and seeded into ~80-160 wells of a 96-wells flat bottom plate. To avoid too sparsely seeded wells, it is advisable to first resuspend in a matrigel volume sufficient to seed 80 wells.

*Therefore, the organoid pellet can be resuspended in approximately 370 (80*4 + 50) μL of 50% matrigel. In case the organoids are too densely seeded, organoids can be diluted by adding more matrigel.*

3. Check the amount of organoids in a 4 μL test drop of matrigel under a light microscope, to determine whether the organoid suspension should be further diluted (the density should be 30-50 structures per drop). Always recheck a new test drop after further dilution.

<u>Note:</u> *when the organoid density is too low, matrigel can be resuspended in 10 mL of Ad-DF+++ and centrifuged at 130 g for 5 min at 4 °C. Next, aspirate medium and resuspend the organoid pellet in less matrigel.*

4. Transfer the organoid suspension to a cold microcentrifuge tube.
5. While preventing bubbles add a 4 μL drop to a (warm) flat-bottom 96-well plate (see Figure 11). This can be performed with a regular p20 pipet.

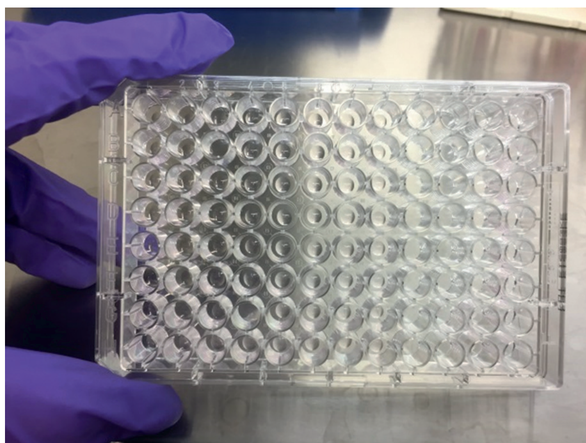


Figure 11. Add 4 μl drops containing 20 - 50 organoids per drop to every well in a pre-warmed 96-wells plate.

<u>Note:</u> *Organoid structures will quickly settle at the bottom of the* microcentrifuge tube *within 30 s so resuspend the organoid suspension with a p200 regularly. While resuspending keep the tube on ice to prevent the matrigel from solidifying in the tube. It is advisable to use a repetitive pipet (like a Viaflo||, Integra) since the organoid seeding is performed more efficient, faster and homogeneously.*

6. Incubate the 96-wells plate for 5-10 min at 37 °C.
7. Add 50 μL of CM +/+ to each well.
8. Incubate 20 -24 h (overnight) at 37 °C, CO_2 5 %.

<u>Note:</u> *Regular organoid passaging and organoid seeding for FIS experiments can be combined. Guideline: prepare excess organoid suspension for seeding in a 96-well plate. Remaining organoid suspension can be diluted at least 1:1 and used for seeding in a 24-well plate for a following 7-day culture.*

C. FIS assay & confocal microscopy (day 2)

1. Incubate Ad-DF+++ at 18 – 37 °C.
2. Turn on the live cell compartment of the confocal microscope and pre-incubate at 37 °C and 5% CO_2 (Pre-heating the live cell compartment takes a minimum of 30-45 min).
3. Prepare fsk and/or CFTR-modulators as desired. Prepare fsk and CFTR-modulator compounds in a 2 x concentration in Ad-DF+++ so when 50 µL is added to the organoids in a well of the 96-well plate containing already 50 µL of CM+/+, , the 1:1 dilution creates a 1 x final concentration of the desired fsk or compound concentration.

Example: if the desired final concentration of fsk within the well is 5 µM, prepare a fsk dilution of 10 µM. If 50 µL of 10 µM fsk is then added to the 50 µL CM +/+, the final concentration will be 5 µM.

4. Prepare 8.4 mM stock of calcein green AM by adding 6 µL of DMSO to one 50 µg calcein green vial. For one full 96 well plate, add 1 µL of 8.4 mM calcein green solution in 1000 µL Ad-DF+++.
5. Add 5 µL of calcein green solution to each well (final concentration is 0.84 µM)
6. Gently resuspend the well 2-3 times with a multichannel for homogeneous staining and efficient uptake of calcein green by the organoid structures. Try tilting the plate and point tips of multichannel in the corner of each well to prevent touching the matrigel drop.
7. Incubate the plate at 37 °C, CO_2 5 % for 15 - 30 min before starting the experiment.
8. Put the 96-well plate in the plate holder of the live cell imaging device and ensure the plate is in fixed position. Live cell imaging settings:

- o Organoid structures with calcein green stain can be visualized upon emission at 488 nm and excitation at 515 nm (detection wavelength range 450 – 700 nm).
- o Use 5 x objectives and ensure an overview of the full matrigel drop by adjusting the focus.
- o Set the position (x, y) and focus (z) of the matrigel drops in the acquisition software.

<u>Note:</u> *an autofocus option may be used when available.*

9. Measurement settings for 60 minute measurement:
   - o Interval = 10 min,
   - o Cycles = 7 (Cycle 1 is t = 0).

Note: the acquisition of images from the total 96-well plate in each time point should not exceed 5 min to ensure optimal comparison of time points between wells.

10. Make sure the calcein green signal in the organoids structures is **slightly oversaturated** and the signal to noise ratio is optimized as much as possible (this is essential for optimal recognition of the organoid structures by the imaging software).
11. Add 50 µL Ad-DF+++ containing fsk stimuli (and/or CFTR-modulators) to each well containing 50 µL of CM +/+. It is preferred to add the stimuli from a 96-well dummy plate with an 8-multichannel for accuracy and efficiency if the size of the live cell chamber allows this. If this is not possible, add the stimuli with a regular pipet.
12. Start the acquisition immediately after stimuli are added.

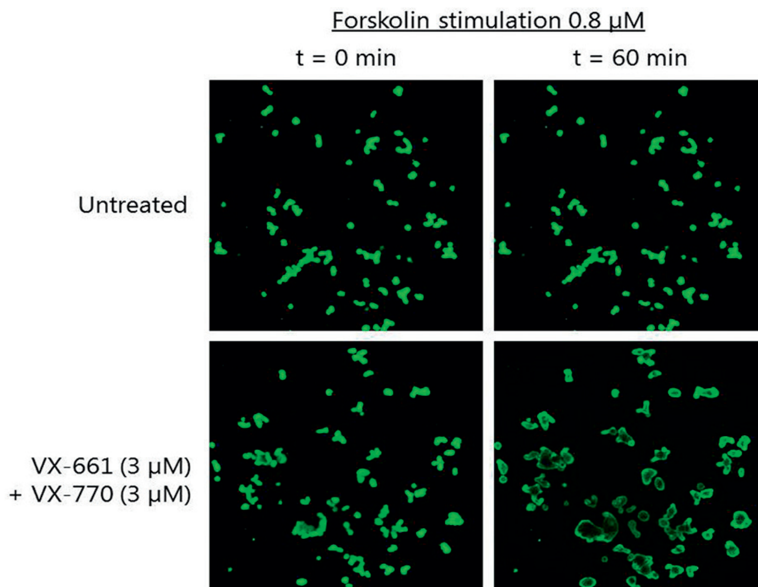


Figure 12. Example of organoid swelling after 60 min FIS assay of F508del/ F508del calcein green stained colon organoids in the absence or presence of VX-661 (3 µM) & VX-770 (3 µM) and fsk (0.8 µM).

European FIS validation & standardization protocol
Timing ~ 8 weeks

To ensure correct implementation of the human organoid technology and the application in the FIS assay, six reference human colon organoid lines (covering different classes of CFTR mutations from severe to mild -see table 5). These organoids lines were tested for basal CFTR function and specific drug response to available CFTR modulators (VX-661 and VX-770). Based on the following protocol, swelling data (technical duplicates at three time points with weekly intervals (n=3)) were generated to compare the qualitative and quantitative results of the implemented organoid technology and FIS assay (not yet published data). These reference organoid lines were selected based on prevalent CFTR genotypes (see table 5) and differences in CFTR basal function and response to CFTR modulators. Researchers that follow the detailed work instructions presented in this protocol should find identical or highly reproducible results (within 10% of AUC values, not yet published data) for these reference lines to confirm successful implementation of the human intestinal organoid technology and FIS assay.

Table 5. Overview of the different organoid cell lines that can be used to validate FIS assay results.

Validation Organoid Cell-lines		
CF class mutation	**Specific genotype**	**HUB ID code**
Class I/ Class I	G542X / G542X	HUB-02-D2-121
Class II/ Class I	F508del / R1162X	HUB-02-D2-038
Class II/ Class II	F508del/ F508del	HUB-02-D2-341
Class II/ Class III	F508del/ G551D	HUB-02-D2-043
Class II/ Class III	F508del/S1251N	HUB-02-D2-103
Class II/ Class IV (mild phenotype)	F508del / R117H - 7T	HUB-02-D2-004

A. Preparations for validation protocol

Medium amounts:

1 liter CM +/+ (see 'Stock preparation of human colon organoid medium components' for instructions on media preparation)

500 mL 2x CM -/-	*Prepare 10 x 100 mL bottles with 50 mL stock and store at -20 °C*
500 mL WCM	*Prepare 2 batches of > 375 mL (> 20 x 145 mm petridishes per batch) and pool.*

0.25 mg R-spo3 — *Stock and aliquot volume dependent on LOT#. Store for 3 months at -80 °C.*

35 mL 50% Matrigel — *Aliquot 2 x 10 mL vials in 500 μL per microcentrifuge tube and store at -20 °C*

B. General experimental set up

The FIS validation assay is based on the experimental set up as previously performed and described (Dekkers et al., 2016, Science).

1. Each reference organoid line was tested using four different conditions (basal CFTR function, VX-661; VX- 770 and VX-661 + VX-770, each in combination with 8 different fsk concentrations (0.008; 0.02; 0.05; 0.128; 0.32; 0.8; 2 or 5 μM) which results in 32 different experimental conditions.
2. Biological replicates for each condition were measured in duplicate and experiments were repeated n=3.
3. Human colon organoids were plated in a 96-well plate as schematically depicted in table 6.
4. VX-661 was added 24 h before the FIS experiment
5. VX-770 was added acutely before the FIS experiment was initiated.

Table 6. Experimental plate set up for measurement of drug response of reference organoid cell lines for validation data.

(Day 1)	**CM +/+**		**CM +/ + + VX-661 3 μM**		**CM +/+**		**CM +/ + + VX-661 3 μM**	
(Day 2)	**Fsk stimuli**				**Fsk stimuli + VX-770 (3 μM)**			
Fsk concentration	0.008 μM	5 μM	0.008 μM	5 μM	0.008 μM	5 μM	0.008 μM	5 μM
	0.008 μM	5 μM	0.008 μM	5 μM	0.008 μM	5 μM	0.008 μM	5 μM
	0.02 μM	2 μM	0.02 μM	2 μM	0.02 μM	2 μM	0.02 μM	2 μM
	0.02 μM	2 μM	0.02 μM	2 μM	0.02 μM	2 μM	0.02 μM	2 μM
	0.05 μM	0.8 μM	0.05 μM	0.8 μM	0.05 μM	0.8 μM	0.05 μM	0.8 μM
	0.05 μM	0.8 μM	0.05 μM	0.8 μM	0.05 μM	0.8 μM	0.05 μM	0.8 μM
	0.128 μM	0.32 μM	0.128 μM	0.32 μM	0.128 μM	0.32 μM	0.128 μM	0.32 μM
	0.128 μM	0.32 μM	0.128 μM	0.32 μM	0.128 μM	0.32 μM	0.128 μM	0.32 μM
	Per organoid reference line							

C. Human colon organoid culturing & workflow (also see table 7)

1. When received, store cryovials with organoid reference lines in liquid nitrogen until ready for the FIS experiments.
2. When ready, thaw a cryovial per reference organoid line as described in thawing protocol A
3. Seed 3 - 4 wells of a pre- warmed 24- well plate for each organoid reference line and add CM +/+ with additional ROCKI.
4. Check the organoid structures by light microscopy after 3 days and ensure there is enough space for the structures to expand. If not consider reseeding the cells with decreased organoid density to allow more space to proliferate and expand.
5. After 7 days of culture check the growth and quality of the organoid structures (compare to structures in Figure 10) :
   a. When the organoids are not yet budding, but appear small and round, densely proliferating organoids: take up and wash the organoids, centrifuge at 130 *g* for 5 min at 4 °C without disruption/shearing and reseed 1 well into 3 new wells to create more space and allow for continuous proliferation of the structures.
   b. When the organoids appear as already “budding” and healthy, large structures: disrupt 1 well according to ‘General handling and passaging of human colon organoid cultures’ (without the cleaning step) and seed into 3-4 new wells.
6. After week 2 and a further 7 days in culture check the appearance of the organoid structures.
   a. When grown into appropriate budding, proliferated organoid structures (see Figure 10): Freeze two full wells into two cryovials according to freezing protocol B . Take the 3rd full well, disrupt and wash the organoids and seed into 3-4 new wells (without the cleaning step).
   b. When not yet grown/proliferated into efficient budding organoid structures and the organoid structures still appear dense and small: repeat the washing and reseeding step without disruption/shearing and seed in 3 wells.
7. After week 3, all organoid lines should be of good culture quality, meaning they should appear as efficiently budding, proliferating structures and sufficient growth. Colon organoids are then ready to be frozen and to start experimental cultures to use in the FIS assay. Make sure a working cell bank is stored for every reference organoid cell line before any FIS experiments are started (see table 7).

8. To start the FIS experiment, expand each organoid reference line into 8 wells in a 24-well plate. This should be sufficient to perform a FIS experiment as well as to reseed organoids for the FIS experiment of the following week. After week 4, plate out organoids to perform the FIS assay. Use the experimental plate lay out as depicted in table 6.

Table 7. Workflow and timelines of FIS assay validation

FIS ASSAY VALIDATION - WORKFLOW & TIMELINES (SUGGESTED)		
Wk nr	**Action**	**Extra comments**
> Wk 0	Prepare all media prior to starting the reference organoid cultures.	
Wk 0	Thaw 1 cryovial into 3- 4 wells (pre-warmed 24-well plate) per reference organoid line.	● Check the seeding density under the microscope.
		● Add ROCKI (10 μM) to CM +/+.
Wk 1	If budding organoid structures are visible:	● Switch to regular CM +/+.
	→ *Disruption 1-2 full well and seed into 4 fresh wells*	● Skip organoid clean up step.
	If organoids are still very small:	
	→ *Take up all 4 wells, centrifuge and seed into 4 fresh wells*	
Wk 2	If budding organoid structures are visible:	● Skip organoid clean up step during passaging.
	→ *Passage 1-2 full wells of organoids into 4 new wells*	
	→ *Pool organoids from 2 wells and freeze a minimum 2 cryovials WCB*	
	If organoids are still very small:	
	→ *Take up all 4 wells, centrifuge and seed into 4 new wells*	
Wk 3	All colon organoid structures should be budding, proliferating structures (Ch. 7, pic 6):	● Skip organoid clean up step during passaging.
	→ *Passage 1-2 full wells of organoids into 4 new wells*	
	→ *Pool organoids from 2 wells and freeze a minimum 2 cryovials WCB*	
Wk 4	Take up 3 - 4 full wells:	
	→ *Follow the organoid disruption & clean up steps.*	
	→ *Seed 8 wells per reference line for FIS assay experiment n=1 in wk 5.*	
Wk 5	Take up 8 full wells:	● Check if confocal settings and image analysis are optimal.

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Table 7. Continued.

FIS ASSAY VALIDATION - WORKFLOW & TIMELINES (SUGGESTED)		
Wk nr	**Action**	**Extra comments**
	→ *Follow the organoid disruption & clean up steps.*	
	→ *Seed 64 wells (in a 96 well plate) per reference line and perform FIS assay experiment n1*	
	→ *Seed 8 wells (in a 24 well plate) per reference line for FIS assay experiment n=2 in wk 6.*	
Wk 6	Take up 8 full wells:	
	→ *Follow the organoid disruption & clean up steps.*	
	→ *Seed 64 wells (in a 96 well plate) per reference line and perform FIS assay experiment n2*	
	→ *Seed 8 wells (in a 24 well plate) per reference line for FIS assay experiment n=3 in wk 7.*	
Wk 7	Take up 8 full wells:	
	→ *Follow the organoid disruption & clean up steps.*	
	→ *Seed 64 wells (in a 96 well plate) per reference line and perform FIS assay experiment n3*	
	→ *Seed 4 wells (in a 24 well plate) per reference line for back up.*	
Wk 7- 8	Data analysis:	● FIS data should be comparable to Dekkers et al (2016).
	→ *Average data of FIS experiments n1, n2 and n3 of all reference lines into n=3*	
	→ *Compare data to FIS results as published.*	
	→ *If finished, stop organoid cultures.*	

D. Preparation of CFTR corrector VX-661 (day 1)

1. Prepare CM +/+ with VX-661 **before** plating out the organoids. VX-661 needs to be added 24 h in advance of the FIS measurement in CM+/+ with a final concentration of 3 µM. For example: from a 20 mM stock: add 95 µL of Ad-DF+++ to 5 µL of 20 mM stock creating 1 mM of VX-661 solution.
2. Per 96-well plate add 9.6 µL of 1 mM VX-661 to 3 mL of CM+/+ creating a final concentration of 3.2 µM.
3. Add 50 µL to each well that requires 24 h of VX-661 incubation (see table 6 for plate lay out)
4. The rest of the wells can be filled with regular CM +/+.

E. Preparation of CFTR stimuli VX-770 & fsk (day 2)

Per 96-well plate:

5. Add 1 µL fsk (10 mM) to 1 mL Ad-DF+++ (= fsk (10 µM)), final concentration in assay will be 5 µM).
6. Add 2 µL of VX-770 stock (20 mM) to 6 mL of Ad-DF+++ (= VX-770 6.4 µM, final concentration will be 3.2 µM.
7. Take 1 mL of VX-770 6.4 µM solution and add 1 µL fsk (10 mM) (= fsk + VX-770 solution)
8. Prepare the titrations (serial dilution) according to table 8 below. Resuspend every suspension 10 times and use a new tip for every dilution step.

Table 8. Preparation of the fsk titration with and without VX-770 added directly before the start of the FIS assay.

Order of addition to plate	Final conc. (µM)	Ad-DF+++ (µL)	Fsk titration (column 1-4)	Ad-DF+++ (µL) + VX770 3 µM	Fsk titration in 3 µM VX-770 (column 5-8)
8	5	-	1000 µL fsk (10 µM)	-	1000 µL fsk / VX-770 mix
7	2	600 µL Ad-DF+++	400 µL from 8	600 µL Ad-DF+++-770	400 µL fsk / VX-770 mix from 8
6	0.8	600 µL Ad-DF+++	400 µL from 7	600 µL Ad-DF+++-770	400 µL from 7
5	0.32	600 µL Ad-DF+++	400 µL from 6	600 µL Ad-DF+++-770	400 µL from 6
4	0.128	600 µL Ad-DF+++	400 µL from 5	600 µL Ad-DF+++-770	400 µL from 5
3	0.05	600 µL Ad-DF+++	400 µL from 4	600 µL Ad-DF+++-770	400 µL from 4
2	0.02	600 µL Ad-DF+++	400 µL from 3	600 µL Ad-DF+++-770	400 µL from 3
1	0.008	600 µL Ad-DF+++	400 µL from 2	600 µL Ad-DF+++-770	400 µL from 2

Expected Outcomes

Sometimes after 3-4 days the colon organoid concentration will be so dense that it is advisable to reseed the structures from 1 well into 3-4 new wells of a 24-wells plate. Take up the small organoids in 1 mL Ad-DF+++ in a microcentrifuge tube, centrifuge at a table top centrifuge and directly plate out in 100 µL fresh matrigel into 3-4 new wells of a 24-wells plate. This allows the small organoid structures to have more space to expand and start budding before they are ready for the first mechanical disruption/ shearing of the organoids.

After 7 days some organoid structures are already efficiently budding and ready for mechanical disruption for splitting. When organoid structures are still very small and not yet efficiently proliferating but the matrigel is fading: do not yet disrupt but take up the small organoids from four wells in 1 mL Ad-DF+++ in a microcentrifuge tube, centrifuge with a table top microcentrifuge and plate the organoids into 100 - 120 µL of fresh matrigel into 3-4 new wells of a 24-wells plate. This allows the small organoid structures to have more time to expand and start budding before they are ready for the first mechanical disruption/ shearing of the organoids.

Quantitative Analysis and Statistics

Raw image data software analysis

The goal of this procedure is to quantify the relative increase in total organoid area (organoid swelling) after stimulation of CFTR function by fsk. The raw data of the FIS assay consists of the confocal images of calcein green stained organoids that are generated at 10 minute intervals, which are digitally stored as a time-series-clip of each well. Image analysis software (e.g. Zen blue (Zeiss), Cell profiler (open-source)) is used to identify the perimeter of each closed organoid structure (X,Y plane), which is defined as a confined region-of-interest. The image software is set to ‘fill objects’ (region-of-interest). Each region-of-interest is expressed as area µm^2 per time point per well. Total area µm^2 associated with all regions-of-interest (organoids) per time point per well is calculated and compared to t = 0 min.

The area µm^2 over a time series of 60 min *(with 10 minute intervals creating 7 time points (t=0; 10; 20; 30; 40; 50; 60 min)* represents the relative swelling increase from baseline. The total increase in area µm^2 during the 7 time points is calculated as percent area increase per well. Hereby t=0 is set as a baseline of 100% and time point t=10 – t=60 min are **normalized** to time point 0 (see table 4). This data can be used for further calculations with the following readouts:

1. Percent increase of organoid area per fsk concentration and condition.
2. The area under the curve (AUC) of the percent increase of organoid area per fsk concentration for all conditions.

Calculations from raw analysis FIS data in Excel

Exported data from imaging software is further processed and calculated in Excel. Data is transposed and calculated in a pre-set format according to the linear trapezoidal method that calculates the average increase over a given time window that is multiplied by the time units within the interval. First, the relative increase over time in area μm^2 per well derived from the exported data is normalized per well to time point 0. Time point 0 is set to 100% and time point 10 – 60 min are normalized accordingly (see table 4, example for 8 measured wells with 4 different conditions; time clips are presented as columns). The relative increase in swelling per well is now expressed in percentages creating 7 data points per well, which can be expressed to show swelling increase per well in time. To more condensely express swelling data (e.g. various forskolin concentrations) in one graph, the area under the curve (AUC) of the relative increase of swelling per well over 60 min can be calculated to create a single data point per well. By using the AUC values, the large datasets can be condensed into graphs capturing all data (see example data in table 4 & Figure 13).

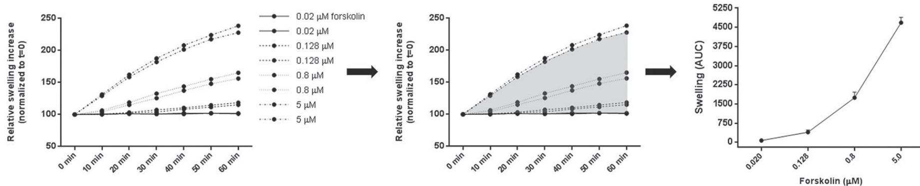

Figure 13. (A) Raw data (area µm2 per well per time point) can be normalized to time point 0 min. Normalized data depicted in a graph shows relative increase in swelling per well over time (baseline is set to 100%). Here, duplicate wells are measured for swelling at 0.02; 0.128; 0.8 and 5 µM fsk for 60 min. (B) The AUC of relative increase in swelling is calculated in Excel. (C) Using AUC calculations for the different conditions, a condensed graph can be created which represents overall relative swelling data per condition over 60 min. Graph C shows the average AUC data of the duplicate wells (from B) with SD.

Table 4.Dissemination of data processing: from raw data to area under the curve (as applied and programmed in Excel).

			Total organoid area µm² (per time point, per well)							
	Calculations		**Well nr 1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Time point (min)			***0.02 µM fsk (duplo)***		***0.128 µM fsk (duplo)***		***0.8 µM fsk (duplo)***		***5 µM fsk (duplo)***	
0	(Raw data)	**1**	281330	238453	263766	188102	331433	321666	300166	314690
10		**2**	283573	239998	263915	189347	345534	342520	388137	413799
20		**3**	284395	241493	270094	194081	381535	382332	476159	510839
30		**4**	283523	240770	274130	201630	416141	427078	546616	590889
40		**5**	283548	243810	285890	207609	455630	463079	604865	654868
50		**6**	287061	243062	294186	215582	490858	499504	653024	705319
60		**7**	286687	241543	303803	223131	517691	531742	684018	750688
	STEP A		**Normalized to time point 0 per well (baseline set to 100%)**							
	(1/1) x 100	**8**	100,00	100,00	100,00	100,00	100,00	100,00	100,00	100,00
	(2/1) x 100	**9**	100,80	100,65	100,06	100,66	104,25	106,48	129,31	131,49
	(3/1) x 100	**10**	101,09	101,27	102,40	103,18	115,12	118,86	158,63	162,33
	(4/1) x 100	**11**	100,78	100,97	103,93	107,19	125,56	132,77	182,10	187,77
	(5/1) x 100	**12**	100,79	102,25	108,39	110,37	137,47	143,96	201,51	208,10
	(6/1) x 100	**13**	102,04	101,93	111,53	114,61	148,10	155,29	217,55	224,13
	(7/1) x 100	**14**	101,90	101,30	115,18	118,62	156,20	165,31	227,88	238,55
	STEP B		**Absolute baseline area**							
	8-100	**15**	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
	9-100	**16**	0,80	0,65	0,06	0,66	4,25	6,48	29,31	31,49
	10-100	**17**	1,09	1,27	2,40	3,18	15,12	18,86	58,63	62,33

Table 4.Dissemination of data processing: from raw data to area under the curve (as applied and programmed in Excel).

			Total organoid area μm² (per time point, per well)							
	Calculations		**Well nr 1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Time point (min)			***0.02 μM fsk (duplo)***		***0.128 μM fsk (duplo)***		***0.8 μM fsk (duplo)***		***5 μM fsk (duplo)***	
	11-100	**18**	0,78	0,97	3,93	7,19	25,56	32,77	82,10	87,77
	12-100	**19**	0,79	2,25	8,39	10,37	37,47	43,96	101,51	108,10
	13-100	**20**	2,04	1,93	11,53	14,61	48,10	55,29	117,55	124,13
	14-100	**21**	1,90	1,30	15,18	18,62	56,20	65,31	127,88	138,55
	STEP C		**Area difference between time points**							
	16-15	**22**	0,80	0,65	0,06	0,66	4,25	6,48	29,31	31,49
	17-16	**23**	0,29	0,63	2,34	2,52	10,86	12,38	29,32	30,84
	18-17	**24**	-0,31	-0,30	1,53	4,01	10,44	13,91	23,47	25,44
	19-18	**25**	0,01	1,27	4,46	3,18	11,91	11,19	19,41	20,33
	20-19	**26**	1,25	-0,31	3,15	4,24	10,63	11,32	16,04	16,03
	21-20	**27**	-0,13	-0,64	3,65	4,01	8,10	10,02	10,33	14,42
	STEP D		**Surface area per 10 minute interval**							
	16 x10/2	**28**	3,99	3,24	0,28	3,31	21,27	32,42	146,54	157,47
	17 x 10 -(**23** x 10/2)	**29**	9,43	9,61	12,28	19,20	96,86	126,72	439,70	469,13
	18 x 10 - (**24** x 10/2)	**30**	9,34	11,23	31,64	51,85	203,37	258,15	703,68	750,50
	19 x 10 - (**25** x 10/2)	**31**	7,84	16,09	61,58	87,81	315,15	383,67	918,07	979,34
	20 x 10 - (**26** x 10/2)	**32**	14,13	20,90	99,60	124,90	427,87	496,25	1095,32	1161,15
	21 x 10 - (**27** x 10/2)	**33**	19,71	16,14	133,56	166,16	521,50	602,98	1227,17	1313,40
	STEP E		**Area under the curve (AUC)**							
Total area	Sum of **28 - 33**	**Y**	**64,4**	**77,2**	**339**	**453,2**	**1586**	**1900,2**	**4530,5**	**4831**

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LIMITATIONS

This protocol aims to provide detailed descriptions on how to establish viable human colon organoid cultures and to reproducible FIS results. The quality and fast processing of rectal biopsies is crucial for successful isolation of stem cell crypts. Despite detailed protocols, quality of the colon organoid media can vary between batches and operators and is mainly caused by the variation in self-produced conditioned media like WCM and NCM. Currently, the activity of essential components such as Wnt-3A and Noggin can be assessed in quantitative assays but organoid responses to these factors may not fully be represented by cell-line based reporter assays which can affect the quality of the colon organoid cultures and outcome of FIS results. Therefore, pictures and examples are included in this protocol to provide guidance for the researcher and allow ongoing monitoring of human colon organoid culture quality and to compare FIS data. Various microscopy platforms can be used, but robustness of FIS data is highly dependent on the quality of the fluorescent images, and especially a low signal to noise ratio leads to unreliable data analysis and non-representative results. Therefore, optimized confocal microscopic procedures and efficient imaging software analysis are needed to ensure capturing high-quality images and calculation of FIS AUC.

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Centralized intestinal organoid generation is a feasible and safe approach for personalized medicine as demonstrated in the European HIT-CF study

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Submitted

ABSTRACT

Introduction: Intestinal organoids show great potential as in vitro drug testing platform for personalised medicine in cystic fibrosis and oncology. Intestinal organoids can be generated by culturing adult stem cells obtained through forceps biopsy or rectal suction biopsy, but the safety of these procedures and the success rates of generating organoids at a centralized lab using these procedures has not been studied in this context. We here report the safety and success rates of both biopsy procedures and subsequent organoid generation in the international multicentre HIT-CF Organoid Study.

Methods: 502 adult patients with Cystic Fibrosis from 33 different hospitals in 12 different countries were included in this study. Based on the preference of the hospital, either forceps biopsies or rectal suction biopsies were obtained and internationally shipped to a central laboratory for organoid generation.

Results: No (serious) adverse events were reported for 280 forceps biopsy procedures, while 222 rectal suction biopsy procedures resulted in 2 (serious) adverse events, namely continued bleeding and a probably nonrelated gastroenteritis. The success rate of organoid generation from all biopsies was 95.0%, and the main reason for failure was insufficient sample viability (3.2%).

Discussion: Our results indicate that both rectal suction and forceps biopsy procedures are safe procedures, and high success rates of organoid generation from the obtained tissue samples demonstrates the feasibility of the organoid technology for personalised *in vitro* testing in an international setting.

INTRODUCTION

Intestinal cell cultures derived from adult stem cells (organoids) are increasingly used to study organ development and diseases such as cancer, inflammatory conditions and inherited diseases [1–5]. Organoids can be expanded almost indefinitely, and maintain functional characteristics of their parental organ [1,6,7]. The genetic stability and long-term expansion allow the generation of organoid collections in biobanks.

Initial studies in Cystic Fibrosis patients [8] demonstrated the direct correlation of the in vitro response of organoids and the clinical response of the same patient to drug treatment. Therefore, we have set up an international effort to determine the use of organoid based personalized in vitro drug testing in individual patients for Cystic Fibrosis. In the international HIT-CF organoid study, rectal biopsies are collected from Cystic Fibrosis patients with ultra-rare genetic mutations. The organoids are tested with a number of clinical phase compounds in central labs [9,10]. The tests will stratify patients for specific clinical drug trials based on the in vitro response.

Intestinal adult stem cells can be harvested from the rectum through forceps or suction biopsy procedures, which are commonly used for other clinical indications and are generally considered safe [11,12]. Nevertheless, the risks of these biopsy procedures and the success rate for generating viable organoid cultures are unknown. In addition, we want to investigate other logistic and technical steps in the organoids generation process that impact the success. Previously, we identified a number of potential challenges such as the number of biopsies, the quality of the biopsies (i.e. viability and presence of stem cells in the obtained tissue), and the transportation time to the specialized organoid generation lab. These factors could impact the success rates of organoid establishment. We here report on the safety of both forceps- and rectal suction biopsies, the feasibility of international transport of biopsies and the success rate of organoid establishment in the European HIT-CF Organoid Study in adults with Cystic Fibrosis [10].

METHODS

Adult patients with Cystic Fibrosis were included in 33 hospitals in 12 European countries for the HIT-CF Organoid Study (NTR7520). This study and the accompanying informed consent form were approved by independent ethics committees at each participating site. Written informed consent (and assent, if appropriate) was obtained from each patient and/or the patient’s legal guardian.

Biopsy collection

Forceps biopsies and rectal suction biopsies were obtained according to detailed working instructions based on a previously published protocol [13]. In short, forceps biopsies were obtained with a flexible endoscope. The endoscope was introduced rectally, and biopsies were obtained on sight, thereby avoiding any arteries or veins. For rectal suction biopsies, patients were first treated with a sodium phosphate enema to cleanse the rectum. Next, the rectal suction device was introduced rectally, and the biopsy opening was positioned dorsolaterally and ~5 cm from the anal verge. To ensure enough material for stem cell isolation, two forceps biopsies or four rectal suction biopsies were obtained from each patient. If the biopsies were deemed to be of insufficient quality, the investigator could decide to obtain more biopsies.

Storage and transport of biopsies

Biopsies were stored in Ad-DF+++ with 0,1% primocin directly after the biopsy procedure and kept at 4 ° C. Detailed information on the composition and the production of this media has been previously published [13]. Within 24 hours of biopsy collection, biopsies were shipped to the central laboratory at Hubrecht Organoid Technology (HUB) in Utrecht, the Netherlands, using a courier service. Biopsies were shipped at 4 ° C. Target delivery time of samples was within 48 hours after biopsy collection.

Crypt isolation and organoid generation

Crypts were isolated according to previously published methods [6,13,14]. In case organoid isolation failed, this was reported to the site and the investigator had the opportunity to re-biopsy a subject. Successful organoid establishment was defined as successful crypt isolation, organoid culturing and subsequent freezing of samples for future use in the study.

RESULTS

Table 1. Subject characteristics and outcomes

	Total population Mean +/- SD (range) or n (%)	Rectal suction biopsy Mean +/- SD (range) or n (%)	Forceps biopsy Mean +/- SD (range) or n (%)
Number of patients (%)	502	222 (54.8)	280 (55.6)
Age (years, mean)	34 +/- 11.5 (16 – 77)	32.0 +/- 10.7 (16 – 67)	32.9 +/- 12.1 (16 – 77)
Sex (female)	257 (51.2)	123 (55.4)	134 (47.9)
Biopsies per patient	3.6 +/- 1.3 (1 – 8)	3.6 +/- 1.4 (1 – 8)	3.7 +/- 1.2 (1 – 7)
Adverse events	2 (0.4)	2 (0.8)	0
Serious adverse events	2 (0.4)	2 (0.8)	0
Gastroenteritis	1	1 (0.4)	0
Hemorrhage	1	1 (0.2)	0
Successful organoid generation	477 (95.0)	214 (96.4)	262 (93.6)
Reasons for failure			
Insufficient sample viability	16 (3.2)	3 (1.4)	13 (4.6)
Contamination of sample	8 (1.6)	4 (1.8)	4 (1.4)
Shipment error	1 (0.2)	1 (0.5)	0

502 adult subjects from 33 different hospitals participated in the study. Gastroenterology departments at all sites were able to perform either forceps biopsy or rectal suction biopsy procedures. Forceps biopsies were performed at 28 sites in 280 subjects, while rectal suction biopsies were performed at 9 different sites in 222 subjects. Four sites performed both procedures.

Two adverse events were reported for the rectal suction biopsy procedures, which were considered serious adverse events. One biopsy procedure led to rectal bleeding which had to be surgically resolved and another associated with campylobacter gastroenteritis, which was probably unrelated to the procedure. No other (serious) adverse events were reported. No other (serious) adverse events were reported.

Overall, 477 organoid cultures were successfully isolated from 502 biopsy procedure (success rate of 95 %). One biopsy sample was lost during shipment due to incorrect storage temperature. A number of samples failed to generate organoids for different reasons. First, several samples were of insufficient viability (16 samples, 3.2 %). Secondly, a number of samples were contaminated during the biopsy procedure (7, 1.4 %) or in the organoid laboratory (1, 0.2 %). The success rate of forceps biopsy procedures was 93.6 %, in comparison to 96.4 % for rectal suction biopsies (Table 1). The majority of samples were processed in the laboratory within 48 hours after the biopsy procedure, while 17 of the 502 samples were processed between 48 and 72 hours after collection, of which 1 failed due to insufficient viability.

DISCUSSION

In our study including 502 subjects from 13 countries and 33 different hospitals, low biopsy complication rates and high success rates of organoid generation were achieved, underscoring the feasibility of international cohort studies using intestinal organoids.

Organoids studies have demonstrated great potential to change the way drugs are developed and clinicals trials can be performed. It has opened up new avenues for personalized medicine and patient stratification in clinical trials [1,2,8]. The novelty of the technology and the requirement of specialized personnel have prompted us to investigate models of organoid application. Here we introduce an international collaboration using a centralized laboratory to implement personalized organoid cultures, which can be particularly relevant in rare diseases with large genetic variability such as Cystic Fibrosis. Our study shows that it is feasible to set up international studies and generate data on a large group of rare patients in centralized labs. The methods used in this study could be implemented for other diseases and efficient shipment of tissue material across countries demonstrates the feasibility of this personalized patient stratification approach for clinical trials, predictive diagnostic tests, and generation of biobanks comprised of internationally obtained samples.

Our study finds very low rates of serious adverse events (<1 %) for both forceps biopsy and rectal suction biopsy, comparable to previous studies [11,12]. These data implicate that both rectal suction biopsies and forceps biopsies are effective tools for obtaining intestinal adult stem cells. Rectal suction biopsy is commonly used by pediatric gastroenterologists to diagnose Hirschsprung, and can be safely performed in newborns [12]. In contrast, rectal suction biopsies are rarely indicated in the adult population which limits the experience of adult gastroenterologists with

this procedure. Ultimately, since both procedures were found to be safe, the choice of biopsy device should be based on the target population and the experience and preference of the operator.

Several examples of organoid biobanks exist, with success rates of organoid establishment ranging from ~60 – 90% [2,15,16]. However, these biobanks were generated from locally obtained samples. Shipment of live tissue samples over large distances requiring several days of transportation was considered to limit the organoid establishment rates. Here we show that standardized working instructions can lead to high success rates in a multicenter setting with extensive transport times of up to 72 hours. The low failure rate of samples processed between 48 and 72 hours after collection suggests that even longer transport times might be feasible. Whether these results can be extrapolated to other organoid models needs to be further investigated.

Interestingly, only 8 samples failed due to sample contamination. Considering, the rectal biopsy procedure and transportation, this demonstrates that the combination of the standardized procedure, media conditions, and transportation allow for efficient collection of clean samples.

In this international, multicentre study, establishment of intestinal organoids from both rectal suction and forceps biopsies was highly successful. These data implicate that international biobanks can be effectively generated through shipment of fresh samples to a central laboratory and support the implementation of these procedures for research and future clinical indications.

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Comparison of organoid swelling and in vivo biomarkers of CFTR function to determine effects of lumacaftor-ivacaftor in patients with Cystic Fibrosis homozygous for the F508del mutation

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TO THE EDITOR

CFTR biomarker assays in patient-derived model systems *in vitro* and in patients *in vivo* enable assessment of response to emerging CFTR-directed therapeutics at the level of the basic CF ion transport defect in individuals with CF and are promising tools to realize the potential of personalized medicine (1, 2). Intestinal organoids represent such a patient-derived *in vitro* model, in which CFTR function can be determined by the forskolin-induced swelling (FIS) assay that sensitively detects functional consequences of CFTR mutations and response to CFTR-directed therapeutics *in vitro*, and correlates with clinical outcomes in patients with CF with a broad spectrum of CFTR genotypes (3–5). Traditional outcome measures of CFTR function such as *in vivo* measurements of sweat chloride concentration (SCC) and nasal potential difference (NPD), and ex vivo intestinal current measurements (ICM) in native tissues cannot be used for preclinical compound testing, but sensitively detect *in vivo* response to approved CFTR modulator therapy (6, 7). Collectively, these studies support that both organoid swelling and *in vivo* biomarkers of CFTR function may be promising tools to determine response to CFTR modulators in individual patients, but their relationships have not been studied. The aim of this study was to compare the effects of the approved CFTR modulator lumacaftor-ivacaftor detected by organoid swelling and *in vivo* CFTR biomarkers, and to determine correlations between paired measurements of organoid FIS with changes in SCC, NPD and ICM in 21 individuals with CF homozygous for the F508del mutation who started treatment with lumacaftor-ivacaftor.

METHODS

This prospective observational study was approved by the ethics committee of the University of Heidelberg. Written informed consent was obtained from all patients participating in the study, their parents, or legal guardians. Clinical outcomes percentage predicted forced expiratory volume in one second ($ppFEV_1$) and body mass index (BMI), and CFTR biomarkers (SCC, NPD and ICM) of 21 individuals with CF homozygous for the F508del mutation were measured before and 8-16 weeks after starting lumacaftor-ivacaftor treatment. Patient characteristics are described in Table 1. Lung function, SCC, NPD and ICM were performed as previously described (6, 8, 9). Rectal biopsies were sent to the University Medical Center Utrecht for organoid generation and FIS experiments using previously described protocols (4). Organoids were pre-incubated (24h) with lumacaftor (3µM VX-809, Selleck Chemicals LLC) and simultaneously stimulated with forskolin (0.128µM) and ivacaftor (3µM VX-770, Selleck Chemicals LLC). Investigators who performed FIS

assays were blinded for patient characteristics. Changes from baseline were tested by paired Student *t*-test and Wilcoxon signed rank test as appropriate. Pearson and Spearman correlation coefficients (r) were calculated to describe relationships between organoid FIS and changes in CFTR biomarkers and clinical outcomes. Correction for multiple comparisons was performed using the Benjamini-Hochberg procedure.

RESULTS

Treatment with lumacaftor-ivacaftor resulted in a clear FIS response in all organoids, albeit with substantial heterogeneity between patients (mean 939.5 ± 234.3, range 541.9 to 1397.6 area under the curve of organoid swelling). In patients, lumacaftor-ivacaftor treatment improved SCC, CFTR Cl^- channel function in intestinal tissues determined by ICM as previously reported (6), whereas no treatment effects were observed in NPD, $ppFEV_1$ or other pulmonary function testing outcomes (Table 1) in this cohort of 21 patients. No correlation was observed between the lumacaftor-ivacaftor induced FIS in organoids versus *in vivo* improvement of CFTR function determined by SCC, NPD or ICM in patients (Figure 1A-C). In addition, neither FIS nor improvement in any of the other biomarkers of CFTR function correlated with improvements in $ppFEV_1$ in CF patients treated with lumacaftor-ivacaftor (Figure 1D).

DISCUSSION

This is the first study to compare effects of CFTR modulator treatment with lumacaftor-ivacaftor on organoid swelling and *in vivo* biomarkers of CFTR function in F508del-homozygous CF patients. Consistent with previous reports, all organoids were responsive to lumacaftor-ivacaftor *in vitro* and ICM improved in all individuals included in our study (Figure 1) (4, 6). However, no correlations were found between the magnitude of FIS of intestinal organoids and changes observed in ICM and other *in vivo* biomarkers of CFTR function (SCC, NPD) (Figure 1). We speculate that this lack of correlation likely results from the limited signal-to-noise ratios determined by the given efficacy of lumacaftor-ivacaftor in F508del-homozygous patients and the variability of the respective CFTR biomarkers. Using SCC, NPD and ICM, we previously demonstrated that lumacaftor-ivacaftor restores ~10-20% of normal CFTR function in F508del-homozygous patients *in vivo* (6). In comparison, previous studies that found high discriminative ability of FIS and correlations with several other biomarkers of CFTR function included patients with a spectrum of CFTR mutations including CFTR gating and residual function mutations and a broader range of responses to different CFTR modulators including highly effective modulator therapy with ivacaftor (3, 4, 5). Based on these previous studies (3, 5), sample size estimation

assuming a nominal type I error of 0.05 and a power of 0.8 suggested a sample size of 20 patients to detect a correlation between FIS and *in vivo* CFTR biomarkers with a correlation coefficient of 0.6. However, in a relatively homogeneous group such as F508del-homozygous individuals with limited response to lumacaftor-ivacaftor, sensitivity may be limited due to measurement variability in both FIS and *in vivo* CFTR biomarkers. Neither FIS nor any of the *in vivo* CFTR biomarkers correlated with changes in $ppFEV_1$ observed after 8-16 weeks of lumacaftor-ivacaftor therapy (Figure 1). Of note, the magnitude of CFTR function detected by FIS and other CFTR biomarkers under lumacaftor-ivacaftor treatment has been associated with milder CF multi-organ disease including pancreatic sufficiency (3, 8). Moreover, biomarkers of CFTR function are probably less dependent on the severity of structural lung damage and rate of decline before the start of lumacaftor-ivacaftor treatment, as well as environmental factors, while these factors can have a substantial impact on $ppFEV_1$ responsiveness. Further, this study did not detect improvement in ppFEV1 at the group level, which may in part be related to the sample size, as well as a larger range in baseline ppFEV1 and shorter duration of this real world observational study compared to the pivotal phase 3 trials (10). Taken together, these findings support that the effects of lumacaftor-ivacaftor detected by FIS and the other CFTR biomarkers are clinically relevant, and that more sensitive outcomes of lung function and structure or longer clinical follow-up may be necessary to unequivocally determine individual benefit of lumacaftor-ivacaftor on clinical outcomes (1, 2).

In summary, our data suggest that FIS of intestinal organoids and *in vivo* biomarkers of CFTR function are sensitive tools for detection and quantification of restoration of CFTR function in response to CFTR-directed therapeutics. However, we did not observe a correlation at the low levels of functional rescue of lumacaftor-ivacaftor in F508del homozygous patients. We conclude future studies in a larger group of patients with a spectrum of responsive CFTR mutations and more effective CFTR modulators (1, 2, 11) will be required to determine the exact role of patient-derived organoids and *in vivo* biomarkers of CFTR function in predicting clinical efficacy of different CFTR modulators in individual patients to enhance precision medicine for CF.

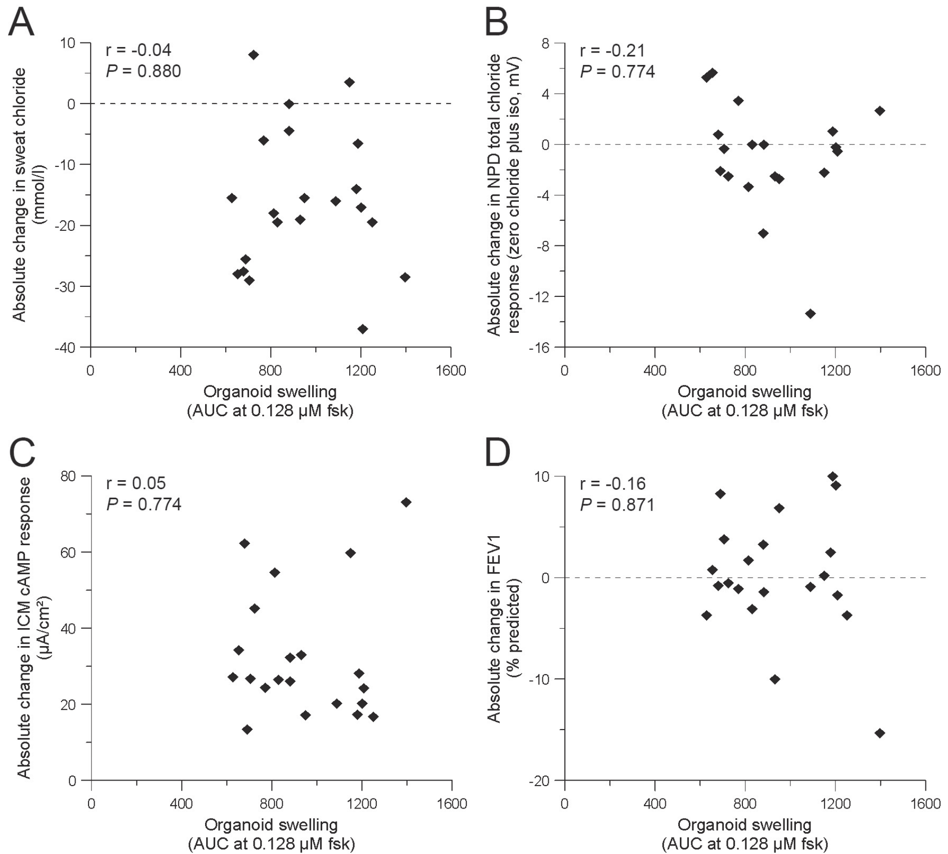

Figure 1: Correlation of effects of lumacaftor–ivacaftor treatment on organoid swelling with effects on in vivo biomarkers of CFTR function and ppFEV1 in F508del-homozygous patients with cystic fibrosis. (A–D) Relationship between forskolin-induced swelling (FIS) of intestinal organoids and absolute change in sweat chloride concentration (A), nasal potential difference total chloride response (zero chloride plus isoproterenol solutions) (B), intestinal current measurement cAMP response (C), and absolute change in ppFEV1 (D). AUC = area under the curve; ICM = intestinal current measurement; iso = isoproterenol; NPD = nasal potential difference. NPD studies were performed in 19 of 21 patients with CF. For panel A, B and D Pearson and for panel C Spearman correlation coefficient r and respective P-values adjusted for multiple comparisons with the Benjamini–Hochberg procedure are provided.

Table 1: Clinical characteristics at baseline and after initiation of lumacaftor–ivacaftor

Clinical characteristic	Baseline mean ± SD (range) or n (%)	Lumacaftor-ivacaftor therapy mean ± SD (range)	Mean change ± SD (range)	p-value
Number of patients	21			
Age (years)	20.4 ± 7.9 (12.0 - 46.0)	20.7 ± 7.9 (12.3 - 46.2)	0.3 ± 0.1 (0.2 - 0.4)	
Sex (female)	8 (38%)			
Genotype F508del/F508del	21 (100%)			
Pancreatic insufficiency	21 (100%)			
Sweat chloride (mmol/l)	88.4 ± 9.2 (69.5 - 109.5)	72.4 ± 13.7 (41.0 - 93.5)	-16.0 ± 11.6 (-37.0 - 8.0)	*<0.001
NPD total chloride response (mV)	0.0 ± 3.5 (-6.8 - 11.6)	-0.9 ± 2.2 (-6.3 - 2.6)	-0.9 ± 4.3 (-13.4 - 5.7)	0.987
ICM cAMP response (µA/cm²)	-5.9 ± 6.6 (-27.5 - 2.4)	26.6 ± 15.7 (3.1 - 68.2)	32.5 ± 16.7 (13.5 - 73.0)	*<0.001
BMI (kg/m²)	19.4 ± 3.2 (15.4 - 26.4)	19.7 ± 3.2 (15.6 - 28.1)	0.3 ± 0.7 (-1.1 - 1.7)	0.287
FEV_1 absolut (L)	2.21 ± 0.68 (0.92 - 3.55)	2.26 ± 0.67 (0.86 - 3.51)	0.05 ± 0.18 (-0.34 - 0.38)	0.987
ppFEV	65.2 ± 19.4 (26.9 - 98.4)	65.4 ± 18.4 (25.2 - 93.8)	0.2 ± 6.0 (-15.3 - 10.0)	0.987
$ppVC_{max}$	81.3 ± 15.1 (44.0 - 100.6)	81.9 ± 14.5 (39.5 - 101.2)	0.5 ± 5.5 (-6.6 - 13.8)	0.987
$ppFEV_1$ /Vcmax	83.1 ± 12.4 (51.1 - 103.3)	82.6 ± 12.1 (48.4 - 105.9)	-0.5 ± 4.4 (-10.5 - 5.8)	0.987
$ppMEF_{25}$	35.2 ± 23.2 (9.0 - 86.0)	35.2 ± 20.7 (9.7 - 82.0)	0.0 ± 11.0 (-29.8 - 18.7)	0.987

Definition of abbreviations: NPD: nasal potential difference; ICM = intestinal current measurement; BMI = body mass index; FEV1 = forced expiratory flow in one second; pp = percent predicted; VC_{max} = maximum vital capacity; MEF_{25} = mean expiratory flow at 25% of capacity. Statistical analyses were performed with paired Student's t-test for sweat chloride, NPD total chloride response, BMI, FEV1 absolut, ppFEV1, ppVCmax, ppFEV1 /Vcmax, ppMEF25 and Wilcoxon signed rank test for ICM cAMP response. *P*-values were adjusted for multiple comparisons with the Benjamini–Hochberg procedure.

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Rectal organoids enable personalized treatment of Cystic Fibrosis

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ABSTRACT

Introduction: *In vitro* drug tests using patient-derived stem cell cultures offer opportunities to individually select efficacious treatments. Here, we provide a study that demonstrates that *in vitro* drug responses in rectal organoids from individual patients with cystic fibrosis correlate with changes in two *in vivo* therapeutic endpoints.

Methods: We measured individual *in vitro* efficaciousness using a functional assay in rectum-derived organoids based on forskolin-induced swelling, and studied the correlation with *in vivo* effects.

Results: The *in vitro* organoid responses correlated with both change in pulmonary response and change in sweat chloride concentration. Receiver operating characteristic curves indicated good to excellent accuracy of the organoid-based test for defining clinical responses.

Discussion: This study indicates that an *in vitro* assay using stem cell cultures can prospectively select efficacious treatments for patients, and suggests that biobanked stem cell resources can be used to tailor individual treatments in a cost-effective and patient-friendly manner.

INTRODUCTION

Functional drug testing on cells or tissue cultures of patients may represent a major step forward for selecting efficacious treatments in an individual setting. Our identification of Lgr5 as a marker of crypt stem cells and the development of technology to grow functional epithelial organoids from such stem cells allows the generation of disease- and patient-specific living biobanks [1–3]. These biobanks could serve as important resources for drug development and scientific studies, but examples demonstrating the validity of these tissue resources for the individual prediction of clinical drug efficacy are currently lacking. Cystic fibrosis (CF) is a genetic disease that is caused by mutations of the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which leads to impaired protein function [4].

Over 2000 CFTR mutations have been identified (www.genet.sickkids.on.ca) and are associated with a variety of clinical phenotypes (www.cftr2.org) [5,6]. Recently developed drugs for CF aim to restore CFTR protein function. Lumacaftor (VX-809) and Tezacaftor (VX-661) are corrector drugs, influencing trafficking of the CFTR protein to the apical membrane, while ivacaftor (VX-770) is a potentiator drug, improving the function of the CFTR protein that is present at the apical membrane. In previous work we showed that also the natural food components genistein and curcumin have potentiator activity *in vitro*, albeit at reduced efficacy and potency as compared to ivacaftor[7]. Currently three CFTR modulating drugs are registered for the treatment of CF patients with specific CFTR mutations; ivacaftor (VX770, Kalydeco®) for patients with different CFTR gating mutations and patients with an R117H mutation, and a combination of ivacaftor and the CFTR correctors lumacaftor or tezacaftor (resp VX770+VX809, Orkambi® and VX770+VX661, Symdeco/Symkevi®) for patients homozygous for the *F508del* mutation and some mutations associated with residual function in the case of Symdeco/Symkevi treatment [8–13].

This CFTR genotype-based stratification for drug prescription presents a challenge for the inclusion of many people with rare CFTR mutations who are not included into clinical trials due to low prevalence of the mutation and lack of mechanistic insights. A recent label extension of ivacaftor by the US Food and Drug Administration (FDA) based on *in vitro* data of heterologous cell lines and mode-of-action, signals a paradigm shift of the regulatory pathway to faster drug access for people with rare CFTR mutations [14]. In previous work we showed that forskolin induced swelling (FIS) of rectal organoids can be used to quantify the function of the CFTR protein in response to CFTR modulating drugs. Forskolin raises intracellular cyclic AMP that

leads to opening of the CFTR ion channel and subsequent ion and fluid transport into the organoid lumen in a CFTR-dependent manner. This readout functionally assesses the impact of both CFTR mutations and additional patient-specific genetic factors that act on CFTR function [15]. In previous work we showed that the *in vitro* response that was measured in rectal organoids correlates with average clinical responses described in patient populations with corresponding genotypes [16]. We also predicted the lack of efficacy of PTC124 (ataluren) in a recent phase 3 clinical trial, by testing of PTC124 in rectal organoids from people carrying nonsense mutations [17,18]. *In vitro* functional testing in rectal organoids of an individual patient may be a next step to facilitate rapid individual access to treatment for patients with rare CFTR mutations.

Currently it is not clear if the *in vitro* FIS response to CFTR modulating drugs correlates with the *in vivo* response at the level of the individual patient. Current clinical outcome parameters and *in vivo* or *ex vivo* biomarkers of CFTR function are highly valuable for measurement of average treatment effects in clinical trials, but they do not correlate at the individual level. A recent meta-study found a small correlation between the *in vivo* pulmonary response and the response of an *in vivo* biomarker of CFTR function (sweat chloride concentration (SCC)), but this study also indicated that individual responses in SCC had a low predictive value for corresponding pulmonary response. Our previous study with rectal organoids showed that two individuals who carried mutations that were not yet characterized, could be successfully selected for a treatment with ivacaftor [16]. We also recently described that FIS measurements of individual patients were related to clinical indicators of CF disease severity, and comparison of FIS and SCC suggested more precise quantification of CFTR function by FIS [19]. We here describe the correlation between the response of FIS of rectal organoids and the *in vivo* therapeutic response for individual CF patients with multiple CFTR genotypes who were treated with several CFTR modulating drugs, and we study the predictive values of the organoid FIS test for the clinical response.

METHODS

Forskolin induced swelling of rectal organoids

Rectal organoids were cultured according to previously described protocols, and are accessible for study by contacting the Hubrecht Organoid Technology foundation (www.hub4organoids.eu) [16,20]. FIS of rectal organoids is a fully CFTR-dependent readout and was measured to indicate baseline CFTR function and response to drugs [15,16]. The organoid response to a drug was calculated by subtracting the dimethyl sulfoxide (DMSO) response at the same forskolin concentration. Organoid

swelling was measured in duplicate at multiple independent culture time points as indicated in supplemental Fig. S1, with 4-8 different concentrations of forskolin as previously described [15,16,21]. The CFTR modulators (3 μM VX-770/ivacaftor (Selleck Chemicals LLC) or a combination of 10 μM genistein (Sigma) plus 50 μM curcumin (Sigma)) were directly added to the organoids with forskolin, except for VX-809/lumacaftor (3 μM, Selleck Chemicals LLC) that was pre-incubated for 24h. Organoids were fluorescently labeled and total area per well and time point was monitored by a Zeiss LSM800 confocal microscope. A Zen Image analysis software module (Zeiss) was used to quantify the organoid response (area under the curve measurements of relative size increase of organoids after 60 minutes forskolin stimulation, t = 0 min baseline of 100%).

Patient selection

A total of 24 patients (15 males and 9 females, median age 16.0 years) were included in this study. From these 24 patients, 15 patients had at least one *S1251N* mutation and were treated with CFTR modulators as part of a clinical trial aiming to compare different CFTR potentiator treatments (NTR4585 and NTR4873). Thirteen of these 15 patients participated in both clinical trials and therefore received two different CFTR modifying treatments. The remaining 9 patients carried at least one rare CFTR mutation and were selected for off-label CFTR modulator treatments based on the organoid response and clinical necessity. A rare mutation was defined as a mutation with a prevalence of less than 1.0 % in the Dutch CF population of which no data on clinical drug responsiveness was available in literature at the time of biopsy [22]. More information on the clinical characteristics of the selected patients is shown in Table 1. All patients (and/or their legal representatives) gave informed consent for rectal biopsies, generating and testing of their individual organoids as well as for (data collection on the effect of) clinical treatment.

Clinical endpoints

In vivo therapeutic effect in the patients with an *S1251N* mutation was measured by absolute change after 8 weeks of CFTR modulator treatment in comparison with pretreatment baseline value. Data from people with rare mutations receiving either ivacaftor or lumacaftor/ivacaftor was collected between 4-8 weeks after initiation of treatment. Forced expiratory volume in one second is a widely used readout to assess pulmonary function, and was expressed as percent predicted for body height, age and gender ($ppFEV_1$). SCC measurements were assessed as this is currently the best established *in vivo* biomarker of CFTR function.

Evaluation of clinical treatment

For all treatments both the patients and those who were involved in clinical data collection were blinded for the magnitude of the *in vitro* drug response of the patients' organoids and vice versa. The $ppFEV_1$ was measured according to ATS-ERS standards [23,24]. The SCC was measured using the Macroduct® system and performed according to the most recent version of the standard operating procedure of the European Cystic Fibrosis Society-Clinical Trial Network.

Quantification and statistical analysis

The primary outcome of the study was the correlation (Pearson) between the *in* vitro organoid and *in vivo* effects (change in $ppFEV_1$ and SCC) plus the predictive capacity of the organoid model, in patients that had a baseline $ppFEV_1$ between 40 and 90 percent. When a change in $ppFEV_1$ or SCC was missing, a patient was excluded from that part of the analysis. In a secondary analysis, we calculated the correlation and predictive capacity for patients that had a baseline $ppFEV_1$ of <40 or >90 percent as well as for the total group of patients that was treated. Finally we used the wilcoxon signed rank test to examined the clinical response of patients with at least one rare CFTR mutation (non- *F508del* or *S1251N*) who had a response in their rectal organoids (area under the curve (AUC) at 0.128 µM forsklin >1000) to the CFTR modulating drug.

Receiver operating characteristic (ROC) curves were generated to evaluate the predictive capacity of organoid FIS for clinical responses. A Youden index was used to select the organoid cut-off point with the most optimal combination of sensitivity and specificity from the ROC-curves [25]. A leave-one-out cross validation further validated our findings [26]. As some patients were treated with two CFTR modifying treatments, we controlled for repeated measurements when calculating correlations and ROC-curves to evaluate a potential bias [27,28]. Because of the limited number of patients, no further subgroup analysis were performed. Statistical analysis were performed using GraphPad Prism 7.02, IBM SPSS Statistics version 22 and R-studio version 0.99.441.

Additional resources

The clinical trial registry numbers and Institutional Review Board (IRB) numbers of the two trials in which the patients with an *S1251N* mutation were treated with genistein plus curcumin and ivacaftor are NTR4585/METC14-268/G-M and NTR4873/METC14-514/M respectively. Additional information on these trials can be found on http://www.trialregister.nl/trialreg/index.asp. The IRB code of the HUB-CF organoid biobank is 14-008.

Table 1: Patient characteristics and treatment regimes

Treatment (duration)	CFTR-genotype	Median age in years at baseline (IQR)	Median ppFEV$_1$ at baseline (IQR)	Median SCC in mmol/L at baseline (IQR)
Genistein plus Curcumin (8 weeks)	*S1251N (p.Ser1251Asn) / F508del (p.Phe508del) n=12*[a] *S1251N (p.Ser1251Asn) / R117H (p.Arg117His) n=1*[a]	15.0 (10.0 – 33.0)	75.5 (64.0 – 93.8)	80.0 (65.5 – 91.0)
Ivacaftor (4-8 weeks)	*S1251N (p.Ser1251Asn) / F508del (p.Phe508del) n=12*[a] *S1251N (p.Ser1251Asn) / R117H (p.Arg117His) n=1*[a] *S1251N (p.Ser1251Asn) / A455E (p.Ala455Glu) n=1* *S1251N (p.Ser1251Asn) / 1717-1G>A (c.1585-1G>A) n=1* *G1249R (p.Gly1249Arg) / F508del (p.Phe508del) n=2* *G461R (p.Gly461Arg) / F508del (p.Phe508del) n=2* *S945L (p.Ser945Leu) / F508del (p.Phe508del) n=1* *R334W (p.Arg334Trp) / R764X (p.Arg764X) n=1* *R553X (p.Arg553X) / 4375-3T>A (c.4243-3T>A) n=1*	16.5 (11.3 – 35.8)	73.0 (59.5 – 94.5)	77.0 (64.0 – 94.0)
Lumacaftor plus Ivacaftor (4 weeks)	*R347P (p.Arg347Pro) / F508del (p.Phe508del) n=1* *W1282X (p.Trp1282X) / F508del (p.Phe508del) n=1*	35.0	30.0	97.0

[a] Patients were treated with both genistein/curcumin and ivacaftor.
CFTR, Cystic Fibrosis Transmembrane conductance Regulator; IQR, Inter Quartile Range; ppFEV$_1$, percentage of predicted forced expiratory volume in one second; SCC, Sweat Chloride Concentration

7

RESULTS

To evaluate the relation between drug response in *in vitro* cultured organoids and therapeutic effect *in vivo*, we studied 37 paired *in vitro-in vivo* responses to three CFTR modulating treatments in 24 subjects with CF (baseline characteristics are provided in Table 1). Fifteen patients with the ivacaftor-responsive *S1251N* mutation received ivacaftor [9]. Thirteen of these patients first received a combination of the possible CFTR potentiating food supplements genistein and curcumin before receiving ivacaftor [7]. The other 9 patients carried at least one rare CFTR mutation with unknown clinical response and were selected for off-label treatment based on the organoid response to either ivacaftor or ivacaftor plus lumacaftor. Apart from the CFTR genotype there were no relevant differences in the baseline clinical characteristics (such as $ppFEV_1$ or SCC values) between patients that received one or two treatments.

We quantified CFTR modulator responses *in vitro* by assessment of FIS of patient-derived rectal organoids that were previously cultured and stored in a biobank (Fig. 1a and 1b show an example, individual measurements for all patients are provided in supplemental Fig. S1). Organoid swelling was assessed after adding various concentrations of forskolin to facilitate optimal detection of drug response across the cohort for the various drugs [15]. We used two outcome parameters to evaluate the *in vivo* clinical effect of a treatment: change in $ppFEV_1$ and change in SCC. Pearson's correlations between organoid response and pulmonary response were analyzed in a subgroup of patients who had a $ppFEV_1$ ≥40% and ≤90% before the start of treatment, to limit non-response of this endpoint (ceiling effects at >90% or irreversible lung damage at <40%), as is usual in clinical trials [9,11,29–32]. The organoid FIS positively correlated with both the pulmonary response (change in $ppFEV_1$; n=21, r=0.610, P=0.003, Fig. 1c) and the change in SCC (n=18, r=-0.762, P=<0.001, Fig. 1d). As observed in other studies with CFTR modulators, the two *in vivo* endpoints appeared only weakly correlated, in a statistically non-significant manner (SCC vs $ppFEV_1$, n=18, r=-0.366, P=0.14, Fig. 1e). We observed no big impact on the correlation of the repeated genistein plus curcumin and ivacaftor measurements; for $ppFEV_1$: n=21, r=0.624, P=<0.001 and for SCC: n=18, r=-0.716, P=<0.001(supplemental Fig. S2). In accordance with previous observations, all correlations were optimal when organoid responses at 0.128 μM forskolin were used (supplemental Table S1) [16]. Patients with a $ppFEV_1$ >90% or $ppFEV_1$ <40% before the start of the treatment did not show a clear correlation between the organoid response and change in $ppFEV_1$, despite an identical correlation between organoids and SCC (Fig. 1f,g)). The data of all patients combined showed correlations of organoids with both ppFEV1 (n=35,

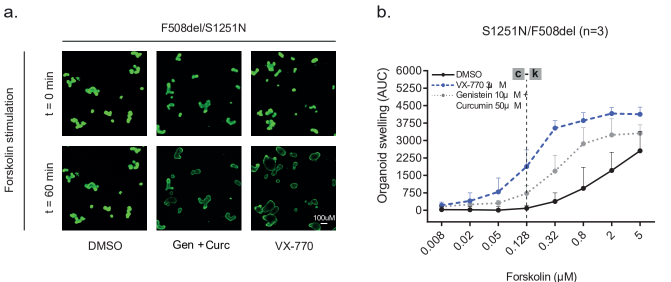
r=0.575, P=<0.001, Fig. 1i) and SCC (n=33, r=-0.708, P=<0.001, Fig. 1j), but a statistically significant relation between $ppFEV_1$ and SCC was not observed. (Fig. 1h,k). People with rare mutations who were selected by organoids prior to treatment showed a median increase of 10% in $ppFEV_1$ (n=7, p=0.058) and a reduction of 39 mmol/L in SCC (n=6, p=0.028). Collectively, these data demonstrates that *in vitro* CFTR modulator responses in organoids correlate with two important therapeutic endpoints.

Prediction of clinical responses using organoids

Next, we generated ROC-curves to examine the predictive potential of different organoid-based thresholds for identifying clinical responders. We dichotomized both the $ppFEV_1$ and SCC response into changes that are generally considered clinically significant and beyond the test variability (changes in ppFEV1>5%, or SCC>20mM or a combined change in $ppFEV_1$>5% and SSC>20mM) and changes that are not [33]. The area under the ROC-curve provides a general measure for test accuracy and was 0.837 (95% CI 0.661 – 1.000) for predicting responders in $ppFEV_1$ and increased towards 0.938 (95% CI 0.830 – 1.000) for predicting responders in either SCC or SCC and $ppFEV_1$ (Fig. 2a). When repeated measurements were taken into account, the area under the ROC-curve did not change. A Youden index was used to select an organoid cut-off point with the most optimal combination of sensitivity and specificity in an unbiased fashion [25]. The selected cut-off value to identify responders in both SCC and $ppFEV_1$ had a sensitivity of 0.80 and a specificity of 1.00 with a corresponding Youden index of 0.8 for identifying responders and non-responders in both $ppFEV_1$ and SCC. The associated positive and negative predictive values were 100% and 80%, respectively. Since data driven selection of the Youden index might cause over-estimation of both sensitivity and specificity, we performed a leave-one-out cross validation to further validate our findings [26]. This additional analysis showed a sensitivity of 0.70 and specificity of 1.00, with a corresponding Youden index of 0.70.

For patients that started with a $ppFEV_1$ <40% or >90% the ROC-curve had an area under the curve between 0.694 and 0.767 (Fig. 2b). For the total group of patients that was treated, the area under the ROC-curve varied between 0.783 and 0.869 (Fig. 2c). Because of the small sample size we did not calculate ROC-curves for the group of patients that had at least one rare CFTR mutation.

In conclusion, the organoid-based test displayed excellent accuracy (AUC of ROC-curve > 0.9) for identifying clinical responses defined by changes in SCC and $ppFEV_1$ or only SCC, while good accuracy (AUC of ROC-curve between 0.8 and 0.9) was observed for identifying clinical responses defined only by $ppFEV_1$ [34].


a.
F508del/S1251N
Forskolin stimulation
t = 0 min
t = 60 min
DMSO
Gen +Curc
VX-770
100uM
b.
S1251N/F508del (n=3)
Organoid swelling (AUC)
DMSO
VX-770 3μM
Genistein 10μM +
Curcumin 50μM
c - k
Forskolin (μM)

≥40% and ≤90%
<40% or >90%
Total
c.
Organoids vs. FEV1 (n=21)
r = 0.610
P = 0.003
d.
Organoids vs. SCC (n=18)
r = - 0.762
P = <0.001
e.
SCC vs. FEV1 (n=18)
r = - 0.366
P = 0.14
f.
Organoids vs. FEV1 (n=14)
r = 0.480
P = 0.08
g.
Organoids vs. SCC (n=13)
r = - 0.695
P = 0.008
h.
SCC vs. FEV1 (n=13)
r = - 0.157
P = 0.61
i.
Organoids vs. FEV1 (n=35)
r = 0.575
P= <0.001
j.
Organoids vs. SCC (n=33)
r = - 0.708
P= <0.001
k.
SCC vs. FEV1 (n=31)
r = -0.198
P = 0.29
Absolute change in FEV1 (%predicted)
Absolute change in SCC (mmol/L)
Organoid swelling (AUC at 0.128 μM fsk)
Genistein + Curcumin in S1251N/F508del
Genistein + Curcumin in S1251N/R117H
VX-770 in S1251N/F508del
VX-770 in S1251N/R117H
VX-770 in S1251N/A455E
VX-770 in S1251N/1717-1G>A
VX-770 in G1249R/F508del
VX-770 in G461R/F508del
VX-770 in R553X/4375-3T>A
VX-770 in S945L/F508del
VX-770 in R334W/R764X
VX-809 + VX-770 in R347P/F508del
VX-809 + VX-770 in W1282X/F508del

Figure 1 - Significant correlation between individual *in vitro* organoid response and *in vivo* change in $ppFEV_1$ and SCC. (a) Confocal images of the FIS of organoids with an *F508del/S1251N* mutation. Images are taken 0 and 60 minutes after adding DMSO, genistein plus curcumin and ivacaftor (VX-770) in combination with forskolin. (b) AUC of the swelling of organoids after measuring for 60 minutes. The graph shows responses after adding eight different concentrations of forskolin in combination with either DMSO or a CFTR modulating treatment. Mean, ± SD (c,d) Pearson correlations between response of the organoids of an individual patient upon CFTR modulating treatment in combination with 0.128 μM Forskolin and the *in vivo* response (change in $ppFEV_1$ and change in SCC) of the same patient to the same treatment for patients who had a $ppFEV_1$ ≥40% and ≤90% before the start of treatment. (e) Pearson correlation between change in $ppFEV_1$ and change in SCC of individual patients upon a CFTR modulating treatment for patients who had a $ppFEV_1$ ≥40% and ≤90% before the start of treatment. (f,g) Pearson correlations between response of the organoids of an individual patient upon CFTR modulating treatment in combination with 0.128 μM Forskolin and the *in vivo* response (change in $ppFEV_1$ and change in SCC) of the same patient to the same treatment for patients who had a $ppFEV_1$<40% or >90% before the start of treatment. (h) Pearson correlation between change in ppFEV1 and change in SCC of individual patients upon a CFTR modulating treatment for patients who had a $ppFEV_1$ <40% or >90% before the start of treatment. (i,j) Pearson correlations between response of the organoids of an individual patient upon CFTR modulating treatment in combination with 0.128 μM Forskolin and the *in vivo* response (change in ppFEV1 and change in SCC) of the same patient to the same treatment for all patients that received treatment. (k) Pearson correlation between change in ppFEV1 and change in SCC of individual patients upon a CFTR modulating treatment for all patients that received treatment. See also Figure S1, S2 and Table S1.

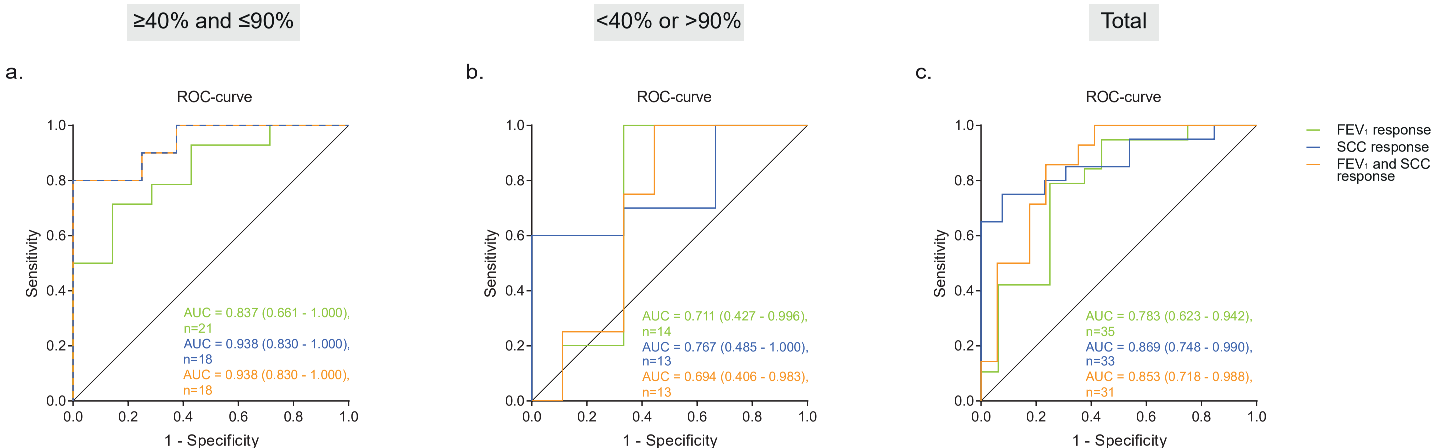


Figure 2 - Predicting individual clinical response by using rectal organoids of a patient. (a) ROC-curves of predicting which patient shows a response in $ppFEV_1$, SCC and both ppFEV1 and SCC for patients who had a $ppFEV_1$ ≥40% and ≤90% before the start of treatment. (b) ROC-curves of predicting which patient shows a response in $ppFEV_1$, SCC and both ppFEV1 and SCC for patients who had a $ppFEV_1$ <40% or >90% before the start of treatment. (c) ROC-curves of predicting which patient shows a response in $ppFEV_1$, SCC and both $ppFEV_1$ and SCC for all patients that received treatment.

DISCUSSION

This study aimed to provide evidence that FIS of rectal organoids can act as a prospective biomarker for *in vivo* CFTR modulator responses. We demonstrated here that individual *in vitro* CFTR modulator responses in these patient-derived stem cell cultures correlate with two independent indicators of therapeutic response *in vivo*. The moderate correlation between FIS and $ppFEV_1$ and higher correlation between FIS and SCC (an *in vivo* biomarker of CFTR function) is in agreement with the higher impact of non-CFTR dependent factors on variation in pulmonary function as compared to SCC [6,35]. We did not find a statistically significant correlation between change in SCC and $ppFEV_1$, probably because of a weaker correlation between these outcome measurements in combination with a small sample size, as was previously also observed in other studies with comparable sample sizes [36]. These *in vivo* endpoints are suitable to indicate treatment effects at a group level, but non-CFTR dependent variation in $ppFEV_1$ and SCC probably limits their precision and accuracy for informing on individual CFTR function modulation.[37] In contrast, *in vitro* FIS is completely CFTR dependent, has sufficient sensitivity to quantitate CFTR modulator activity and the repeated measurements increase precision. These properties likely facilitate that FIS has sufficient accuracy to inform on both $ppFEV_1$ and SCC (or their combination), suggesting that FIS is a potent biomarker to quantitate individual CFTR modulator responses.

Our dataset provides a first analysis of the predictive potential of the rectal organoids to identify clinical responders and non-responders to treatment. Our data support that FIS can be used to prospectively select responders and non-responders to CFTR modulator treatments but the cut-off value with the highest Youden index still needs to be interpreted carefully as well as the definition of clinical responders. The Youden index selects the most optimal ratio between sensitivity and specificity, but a different threshold with a higher negative predictive value may be preferential to limit the exclusion of treatment responders (e.g. an organoid threshold with a negative predictive value of 100% would have a positive predictive value of 77%). Additionally, it remains unclear how short term treatment responses individually translate into long term clinical response. It could therefore be that the definitions for long term clinical responders are different, leading to other threshold values of predictive tests. We observed that the correlation of the organoid test with response in $ppFEV_1$ was modified by baseline $ppFEV_1$, despite similar correlation in SCC in both groups with differences in baseline $ppFEV_1$. This supports that biomarkers of CFTR function such as organoid-based measurements have an important role for assessment of CFTR

modulator responses in subjects where clinical domain indicators are unsuited to measure therapeutic response.

There are several limitations in this study. First, the open-label setting of treatments can induce bias in the acquisition of clinical data. Potentially $ppFEV_1$ might have been influenced, but this is unlikely for SCC measurements. However, we do not expect that the open label setting has strongly affected the *in vitro-in vivo* correlation, since the clinical observers and patients were blinded for the *in vitro* drug responses and vice versa. Second, the study is biased for potentiator treatments. The area under the ROC-curves may be different when patients are stratified for different CFTR modulator treatments such as corrector/potentiator combinations. Also the cut-off values of $ppFEV_1$ and SCC that were used to define a clinical responder may not be fully accurate in identifying long-term clinical responders to treatment, and changing these cut-off values will lead to different ROC-curves. Third, patient subgroups with differences in organoid baseline CFTR functions may require different organoid test conditions (e.g. different forskolin conditions) for better predictive values. Fourth, it remains challenging to estimate adequate drug concentrations in the organoid tests as to optimally reflect the *in vivo* tissue concentration. For ivacaftor and lumacaftor we relied on average blood concentrations to determine the *in vitro* drug concentrations [38,39]. For genistein and curcumin, lack of information on *in vivo* tissue concentrations may resulted in overdosing the *in vitro* situation, which can lead to overestimation of their potential *in vivo* effect. Most importantly, larger follow up studies remain needed to define more precisely how organoid-based measurements, and possibly other short term endpoints, can predict long term individual benefit to various CFTR modulator treatments.

Apart from the performance of FIS as a biomarker of treatment response in this study, the rectal organoids provide additional benefits over other biomarkers of CFTR function. Rectal organoids are adult stem cell cultures that can be generated from a single rectal biopsy and cultured over 6 months while maintaining patient-specific CFTR modulator response [16,40]. Rectal biopsies are accessible in most subjects independent of age and can be shipped to dedicated centers for organoid testing within weeks and stored in living biobanks which enables future drug testing [16]. The FIS readout appears also not affected by CF disease phenotype (e.g. irreversible damage and inflammation in pulmonary markers). Currently, the immediate impact can be the selection of people for treatments independent of the CFTR genotype, both for CFTR modulators on the market and in development. For people having access to treatment, we may be able to further individually tailor treatments to maximize clinical benefits [41].

CONCLUSION

In vitro drug efficacy measurements by FIS in rectal organoids of individuals with CF correlate with the most important *in vivo* response indicators of CFTR modulators (change in $ppFEV_1$ and SCC). The data further suggest that thresholds can be established to prospectively identify clinical responders with acceptable positive and negative predictive values. Organoid testing can provide a patient-friendly and cost-effective approach to increase access to treatment for patients with CF, and optimize risk-benefit and cost-effectiveness of treatments. This study is a first example that *in vitro* tests using cultures of patient stem cells, stored in a living biobanks, can be used to predict individual treatment benefits.

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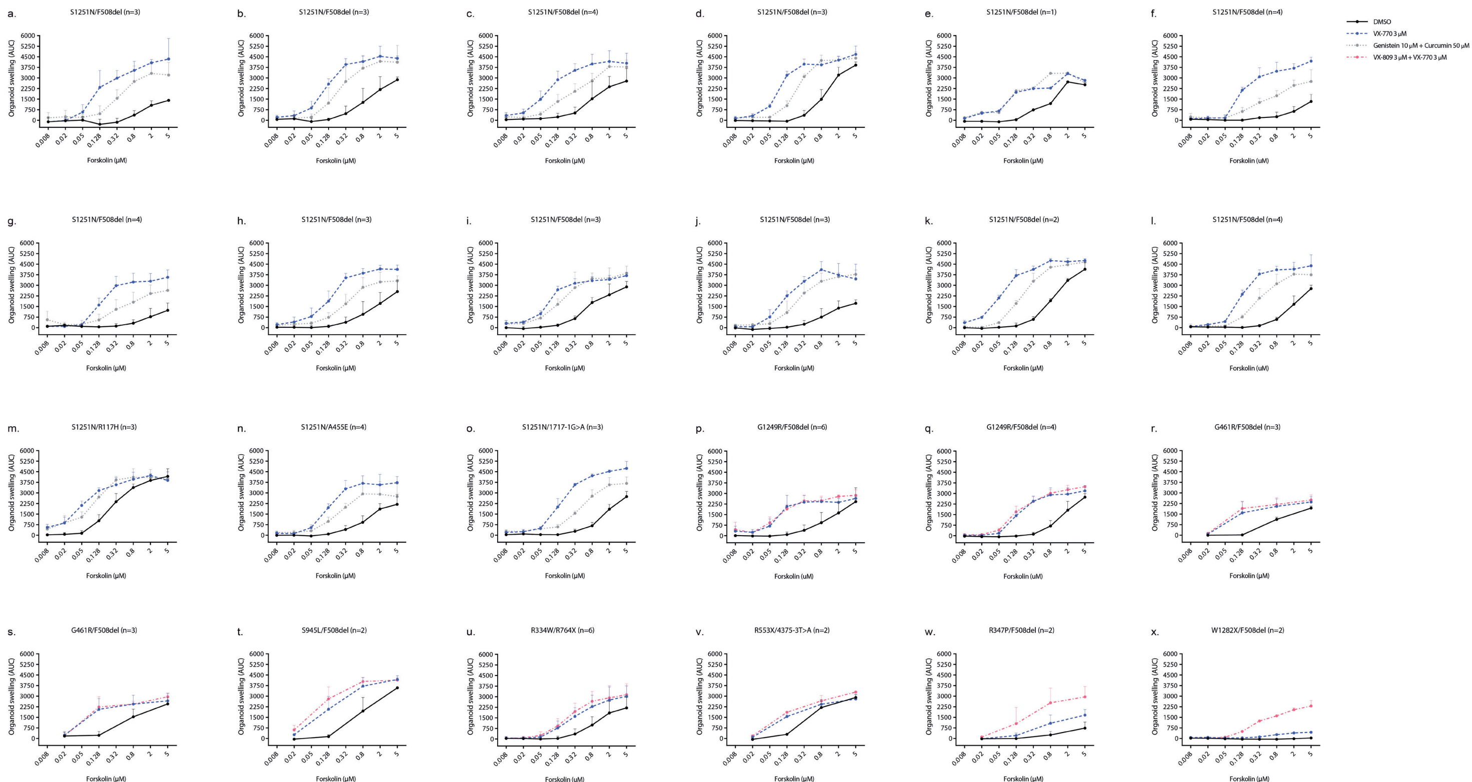
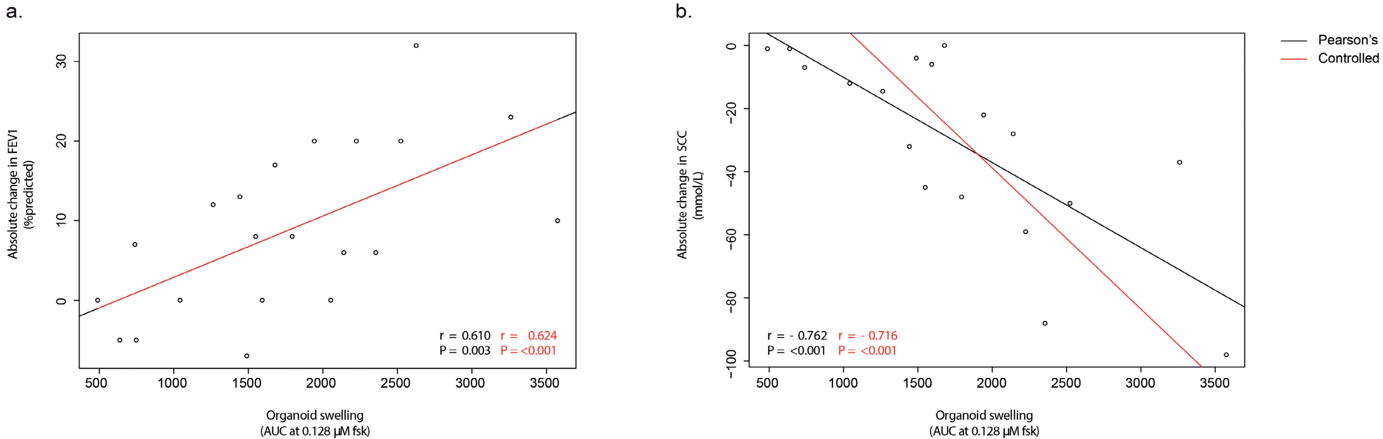
SUPPLEMENTARY DATA



Supplemental Figure S1. - Individual organoid measurements of all patients that received treatment (a-x) Area Under the Curve (AUC) of the swelling of organoids of all individual patients after measuring for 60 minutes. The graphs show responses after adding four to eight different concentrations of forskolin in combination with either DMSO or a CFTR modulating treatment. Mean, ± SD. (h) Was also used as an example in Fig 1b.



Supplemental Figure S2. Correlation between individual in vitro organoid response and in vivo change in $ppFEV_1$ and SCC, controlled for repeated measurements. (a,b) Correlations between response of the organoids of an individual patient upon CFTR modulating treatment in combination with 0.128 µM Forskolin and the in vivo response (change in $ppFEV_1$ and change in SCC) of the same patient to the same treatment for patients who had a $ppFEV_1$ ≥40% and ≤90% before the start of treatment, controlled for repeated measurements.

Supplemental Table S1. Correlation of *in vivo* and *in vitro* response with different forskolin concentrations

Forskolin concentration (µM)	Organoids vs. $ppFEV_1$			Organoids vs. SCC		
	n	*r (95% CI)*	*p-value*	*n*	*r (95% CI)*	*p-value*
0.050	20	0.294 (-0.170 - 0.652)	0.21	17	-0.417 (-0.748 - 0.080)	0.10
0.128 (fig 1c,d)	21	0.610 (0.242 - 0.825)	0.003	18	-0.762 (-0.906 - -0.458)	<0.001
0.320	20	0.407 (-0.044 - 0.720)	0.08	17	-0.766 (-0.911 - -0.452)	<0.001
0.800	21	0.065 (-0.378 - 0.483)	0.78	18	-0.580 (-0.824 - -0.155)	0.01

$ppFEV_1$, percentage of predicted forced expiratory volume in one second; SCC, Sweat Chloride Concentration

Supplemental Table S2. Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Human rectal tissue	This paper http://hub4organoids.eu/	
Chemicals, Peptides, and Recombinant Proteins		
B27 supplement with Vitamin A	Thermo Fisher Scientific: Invitrogen	Cat# 17504-044
N-Acetylcysteine	Sigma Aldrich	Cat# A9165-25g
Nicotinamide	Sigma Aldrich	Cat# N0636
Mouse Epithelial Growth Factor	Invitrogen	Cat# PMG8043-1mg
TGFb type I Receptor inhibitor (A83-01)	Tocris	Cat# 2939
p38 MAPK inhibitor (SB202190)	Sigma Aldrich	Cat# S7067-25mg
Calcein, AM	Life Technologies: Gibco	Cat# C3100MP
Forskolin	Sigma	Cat# F3919-10mg
Lumacaftor (VX-809)	Selleckchem	Cat# s1565
Ivacaftor (VX-770)	Selleckchem	Cat# s1144
Genistein	Sigma	Cat# 92136-10mg
Curcumin	Sigma	Cat# C7727-500mg
Deposited Data		

Supplemental Table S2. Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CFTR2 database	Johns Hopkins University / Hospital for Sick Children / CF Foundation	https://www.cftr2.org/
Experimental Models: Cell Lines		
Human rectal organoid lines	This paper http://hub4organoids.eu/	
L- Wnt 3A producing cell line	http://hub4organoids.eu/	
Hek293T – Noggin hFc cell line	http://hub4organoids.eu/	
Hek293T – R-spondin-1 mFc cell line	Trevigen	Cat# 3710-001-K
Software and Algorithms		
Zen Image analysis software module	Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen/image-analysis.html
SPSS	IBM	https://www.ibm.com/analytics/nl/nl/technology/spss/
R-studio	https://www.rstudio.com/	https://www.rstudio.com/
Graphpad prism	Graphpad	https://www.graphpad.com/scientific-software/prism/
Other		
Matrigel ® (protein concentration between 9.8-10.2 mg/ml)	Corning	Cat# 354230

Rationale and design of the HIT-CF organoid study: stratifying Cystic Fibrosis patients based on intestinal organoid response to different CFTR-modulators

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ABSTRACT

Background: Cystic fibrosis is a rare recessive monogenic disease caused by loss-of-function mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* (CFTR) gene. Recently developed CFTR-modulators (i.e. drugs that rescue CFTR-function) have improved the prognosis of the disease, but are only available for certain, relatively common mutations. More than 2000 genetic variants in CFTR have been described, many of which are uncharacterized, while efficacy of CFTR-modulators can differ depending on genotype. Personalized models that can predict drug efficacy in patients with rare mutations could help to provide access to effective CFTR-modulator treatment. The forskolin induced swelling (FIS) assay in rectal organoids can robustly measure CFTR-function and CFTR-modulator efficacy, and pilot studies indicate that FIS accurately predicts treatment efficacy in individual patients. In the HIT-CF Organoid Study, rectal organoids from patients with rare CFTR-mutations are screened for CFTR-modulator efficacy to identify patients that could clinically benefit from these drugs.

Methods: In this EU-H2020 funded multi-centre study, rectal biopsies are obtained from approximately 500 subjects with rare CFTR-mutations from 17 different EU countries and sent to central laboratories. Organoids are generated, on which several novel CFTR-modulating drugs are screened for efficacy. In the process, a biobank is generated which could be used for future research. Subjects whose organoids show a response to treatment will be asked to participate in subsequent clinical trials evaluating the clinical efficacy of tested drugs.

Discussion: The HIT-CF Organoid Study applies the intestinal organoid model for personalized medicine in patients with rare variant cystic fibrosis, who are currently excluded from classical CFTR-modulator trials. Together with the subsequent clinical trials in high responders, this study aims to create a new pathway for access to CFTR-modulating drugs for patients with ultra-rare CFTR variants.

BACKGROUND

Cystic Fibrosis (CF) is a life shortening rare disease caused by loss-of-function mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene (1,2). Dysfunctional CFTR impairs epithelial chloride and bicarbonate transport, which leads to thickening of secretions in multiple organs, resulting in respiratory failure, endocrine dysfunction, liver cirrhosis, gastrointestinal disorders and early death [3]. CF is a monogenic recessive disease, and more than 2000 different variants in the *CFTR* gene have been described(genet.sickkids.on.ca) [4]. The F508del mutation is present in ~80% of European CF patients, but 351 other variants have been confirmed as pathogenic [5]. The characteristics and disease liability of the remaining variants remain mostly unclear. They can affect CFTR function via distinct (combinations of) mechanisms and can result in different levels of CFTR dysfunction and clinical disease [1,6].

The recent introduction of so-called CFTR-modulators has caused a paradigm shift in the treatment of cystic fibrosis. Four different drug categories can increase CFTR protein availability and function, and act in a *CFTR* mutation specific fashion. Ivacaftor, the first CFTR modulator on the market, was identified as CFTR potentiator for patients with so-called gating mutations that are present in ~5% of CF patients. In these patients ivacaftor increases the open-probability of the CFTR channel, thereby improving the anion transport [7].

A combination of ivacaftor and the corrector lumacaftor proved to be effective in patients with the most common F508del mutation [8,9]. CFTR-correctors aim to correct impaired CFTR folding and trafficking to the cell surface [10,11]. Typically a potentiator with one or two correctors are needed to optimally re-establish the function of the complex CFTR-channel in patients with one or two F508del alleles [12,13], and these combination treatments can lead to effective treatment for ~85% of the CF population [14,15]. The third type of CFTR modulators are CFTR-read-through agents that promote ribosomal read-through at premature termination codons (PTCs) and thus lead to full length CFTR protein production in patients with nonsense mutations [16]. CFTR-amplifiers increase CFTR mRNA availability and might act in a genotype agnostic fashion [17]. The therapeutic targets of read-through agents and CFTR-amplifiers still need to be assessed. Another interesting CFTR-modulating drug category are CFTR-stabilizers that aim to stabilize CFTR-protein activity at the cell membrane through different molecular targets [18,19]. CF patients with non-F508del and non-gating mutations (approximately 12000-13000 patients worldwide) are very heterogeneous and carry >2000 different ultra-rare and mostly uncharacterized *CFTR* mutations

(genet.sickkids.on.ca and cftr2.org) [4,5]. Moreover, patients carry a combination of two *CFTR* variants which further complicates the prediction of drug efficacy in individual patients. The role of current CFTR-modulators in these patients is hardly described and clinical efficacy can not be tested in classical clinical trials due to extremely low patient numbers. In this population, there is a clear unmet need to match the best CFTR-modulator (combination) to each individual.

Recent stem cell technologies enable the long-term culture of patient-derived tissues in the laboratory as organoids [20,21]. Organoids are three-dimensional cell cultures that recapitulate features of the tissue from which they originate [20]. Dekkers et al developed a functional CFTR assay in intestinal organoids that were grown from easily obtainable rectal biopsies which provides a way to rapidly collect and biobank tissues from individual patients [22]. CFTR function can be measured upon stimulation of organoids with forskolin, which leads to CFTR phosphorylation, CFTR channel opening and subsequent transport of chloride and water into the organoid lumen. Although forskolin-induced swelling (FIS) of intestinal organoids is an indirect measurement of CFTR function as it quantifies fluid rather than ion transport, FIS is fully CFTR dependent and provides a rapid, phenotypic assessment of CFTR function. Residual CFTR function in organoids correlates with several clinical disease parameters [23,24] and biomarkers of CFTR function [25].

Importantly, the effect of CFTR modulators on CFTR function can be robustly measured by the FIS assay through quantification of increased swelling upon drug incubation *in vitro* [26]. Using this assay, correlations were observed between organoid drug-induced swelling and drug responses in clinical trials at the population level [26], as well as a high-to-excellent accuracy predicting individual *in vivo* responses to CFTR modulators [27]. This has led to the identification of successful treatment of several patients with ultra-rare genotypes that showed high FIS to CFTR-modulating drugs in their organoids [26,27]. Moreover, drugs that showed potential in other pre-clinical model systems but not in organoids subsequently failed to produce clinical benefit, highlighting the specificity of organoids in finding meaningful clinical changes [28]. By providing an individual assessment of CFTR modulator efficacy without a priori knowledge of the *CFTR* mutations, organoid CFTR function might be exceptionally suited to stratify people with ultra-rare mutations for effective treatments. To further implement the FIS assay as a personalized medicine approach for patients with rare CFTR-mutations, we developed the HIT-CF project (www.hitcf.org) [29].

Rationale for the current study

The Human Individualized Treatment for CF (HIT-CF) project was developed to bring CFTR-modulators to CF patients with rare mutations, and has received funding through the European Union's Horizon2020 programme (grant agreement No. 755021). The project consists of three parallel and intertwined pathways aiming to generate new treatment options and reimbursement of drugs for this patient group (Figure 1). First, the HIT-CF Organoid Study will screen different CFTR-modulators on organoids generated from 500 patients with rare *CFTR* mutations across Europe. Multiple pharmaceutical companies have committed to providing their investigational drugs (currently in phase I/II clinical trials) for the screening phase. The compounds being tested comprise the full range of different CFTR-modulator categories currently in the clinical domain (i.e. read-through agents, amplifiers, correctors and potentiators). An *in vitro* drug screening will identify which patients are most likely to clinically respond to one of the drug(s) (combinations). Once these patients have been identified, subsequent studies will evaluate the clinical efficacy of the drugs in patients identified as being '*in vitro* responders', with the aim to gather sufficient data for regulatory approval of the drugs in these specific patients.

The second pathway is the generation of a living organoid biobank. Organoids from all 500 subjects in the HIT-CF Organoid Study will be stored for the study at Hubrecht Organoid Technology (HUB), and can be used for future drug development efforts. Currently, a biobank is set up to include materials from subjects across Europe. When patients consent to the long-term storage of their organoids, these can be used by academic laboratories worldwide, and collaborations can be explored with pharmaceutical companies to screen potential new CFTR-modulating drugs in this patient population.

The third pathway is aimed at regulatory access and reimbursement of the drugs in this patient population. Within the project, organoids are used to identify patients and data will be gathered on the predictive value of organoids. If this approach is to be implemented in regular practice, it needs the support of stakeholders such as regulatory agencies and insurance companies. Discussions with these stakeholders are ongoing to clarify what data is important, and their advice will be used to further optimize the project. The currently ongoing HIT-CF Organoid Study (NTR7520) will be described here.

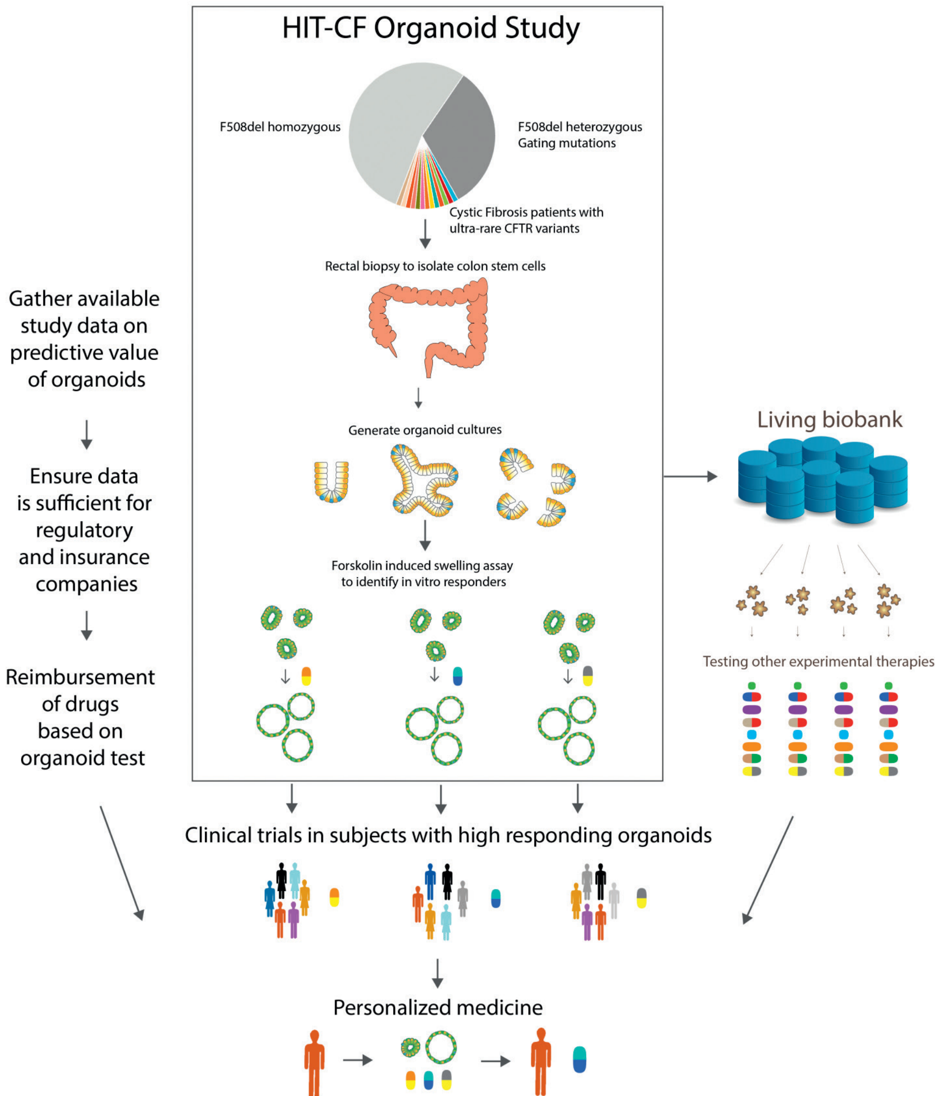

Figure 1. Overview of the HIT-CF Project

METHODS

Clinical design

The aim of the HIT-CF Organoid Study is to collect rectal biopsies and culture intestinal organoids from at least 500 patients with ultra-rare *CFTR* mutations on both

alleles. Subsequently the study aims to identify the predicted best clinical responders (based on amount of organoid swelling) to new CFTR-modulators from different pharmaceutical companies. The study design is an international multi-center study. Rectal biopsies are collected from 500 subjects in different clinical sites across Europe, all members of the clinical trial network of the European Cystic Fibrosis Society (ECFS-CTN). Investigational study drugs will be tested on each organoid in one of three academic laboratories (University Medical Centre Utrecht (UMCU), Katholieke Universiteit Leuven (KUL) and the University of Lisboa (LIS)). A subset of responsive organoids in the academic laboratories will be further screened at HUB and ranked according to level of response to one of the lead drug combinations from each pharmaceutical partner. The subjects with the highest organoid responses will then be asked to participate in subsequent clinical trials. For more details see 'organoid culturing and drug screening' below.

Patient population

The target population is male and female adult (older than 16 or 18 years depending on country-specific ethical regulations)subjects diagnosed with Cystic Fibrosis with a sweat chloride concentration >60 mmol/L, without a mutation for whom CFTR-modulating drugs are currently available (homozygous or heterozygous F508del mutations or gating mutations), and without combinations of frequently occurring class I mutations (1717-1G>A, 621+1G>T, 3120+1G>A, 1898+1G->A, CFTRdele2,3 and 2183AA->G). Patients that have a comorbidity that (to the opinion of the investigator) might pose a risk while participating in a clinical trial will be excluded from study participation. Moreover, patients that have had a lung transplantation are also ineligible.

Sample size

The study goal is to identify responders that are eligible for subsequent HIT-CF clinical trials. Our preliminary power calculations for the clinical trial with lung function (FEV1) as the primary endpoint, indicate a power of >80% if 16 subjects are included. Therefore the study aims at recruiting 20 patients to mitigate drop-out during the study. This sample size is comparable to a previously published trial in a similar patient population and intervention [30]. As we expect that some patients will not be eligible for a clinical trial, we aim to identify 27 patients per trial eligible for recruitment. This will allow us to contact a new subject if a subject is not eligible to participate. The inclusion rate for the clinical trial needed to include 20 out of 27 patients is thus 74%. To include 16 evaluable subjects the minimum needed inclusion rate is 55%. In experiments performed so far on ultra-rare organoids, we have observed that ~16% of them react to the CFTR-modulating drugs ivacaftor

or lumacaftor/ivacaftor, resulting in about 80 *in vitro* responders in the observed population of 500 subjects, which covers about 27 subjects for each of the three intended clinical trials.

Study procedures
The study procedure performed is collection of rectal biopsies by either forceps during sigmoidoscopy or using a rectal suction device. These are not part of regular medical treatment. Each site has the possibility to choose the procedure they are most experienced in. The success rate of generating organoids out of two forceps biopsies is >92%. Success rates for rectal suction device is >95% if four biopsies are obtained, and ~80% if two biopsies are obtained (unpublished data from UMCU/HUB). Therefore, either two forceps or four rectal suction biopsies will be obtained per subject. If no organoids can be generated, the patient will be re-contacted and asked whether they are willing to undergo another biopsy. The biopsies will have no influence on regular medical care. If intestinal organoids have already been generated for a subject in a previous study or for routine medical care and can be made available for testing, subjects do not need to undergo the study procedure again.

Rectal suction biopsy
Biopsies can be collected using a rectal suction device. This device is used to facilitate diagnosis of the disease Morbus Hirschsprung, and to obtain material for Intestinal Current Measurement for the diagnosis of Cystic Fibrosis. Therefore, many CF-centres have experience with rectal suction biopsies. A working instruction is provided to the sites with detailed instructions how to perform the procedure.

To obtain the best possible biopsies, patients will be treated with an enema (sodiumphosphate), to clean the most distal part of the colon. If this is contraindicated, biopsies will be collected without a preceding enema. During the procedure, patients will lie on their side, with knees pulled upwards towards the chest. Next, the rectal suction device is introduced rectally, and inserted ~6 cm. The rectal suction device is connected by a rubber tube to a vacuum meter, and the vacuum meter is attached by a rubber tube to a 50cc plastic syringe. The assisting nurse sucks air into the suction device by pulling on the plunger of the syringe. This creates a vacuum, and intestinal tissue is sucked into the suction device through an opening with a diameter of 2.0 millimeter. This tissue is subsequently cut by the rectal suction device, and the tissue is collected. The diameter of the collected tissue is around 2 mm, and less than 1 mm thick. The biopsies are taken on the dorsolateral sides of the rectum, because no pain receptors are present there and no major blood vessels

are present. It is important that the device opening has passed the anal sphincter to avoid pain. Collecting organoids using a rectal suction device has a very low risk of complications. Friedmacher et al. have recently published a systematic review, in which they found that the risk of any complication is 0.63%. Persistent rectal bleeding was the most common complication with an incidence of 0.5% [31]. The article states that in very young children (<1 year of age), the risk is relatively higher. We will perform this procedure in adult subjects, and subjects are instructed to call the hospital if bleeding is persistent or if they have any other complaints. To detect any prolonged bleeding and intervene as early as possible, patients will stay in the hospital for one hour after the procedure.

Forceps biopsy

Biopsies can be collected by forceps during rectoscopy. This device is used by trained gastro-enterologists to obtain material for a wide spectrum of diseases. The procedure is done endoscopically with a flexible endoscope. The endoscope is introduced rectally. The biopsies are obtained on sight, thereby avoiding any arteries or veins. In a recently published article, the safety of this procedure was evaluated by studying 353 forceps biopsies. No major complications were reported. Only one patient complained of abdominal pain following the procedure, but fully recovered after 4 hours observation [32]. Patients are observed after the procedure to quickly notice any possible complications.

Generating organoids from rectal biopsies

At the HUB organoids will be generated from the rectal biopsies using the protocol as previously published [22,33,34]. If organoids are received from study sites, these will be further cultured to ensure viability of material. From HUB, organoids will be transferred to the Cystic Fibrosis Research Laboratory of University Medical Center Utrecht, the Organ Systems Laboratory of KU Leuven and the Cystic Fibrosis Research Laboratory of the University of Lisbon. Samples will be transported by a specialist courier service.

Organoid culturing and drug screening

UMCU protocols for CFTR function measurements have been established under ISO-9001 conditions and HUB has ISO-270001 compliant protocols. The most updated protocols of UMC and HUB have been combined and shared with the laboratories in Leuven and Lisbon for preparation of organoid culture media, organoid growing, and CFTR function measurement by forskolin-induced swelling (FIS) [33]. From these, one standard lab manual has been generated for all the labs to make sure the procedures are standardised. Additionally, identical lot numbers

and preferably batches of reagents will be obtained by all labs to ensure the most optimal standardisation.

Drugs to be tested

The screening of the organoids will be divided up based on which drugs are being tested. Flatley Discovery Labs (FDL) is developing CFTR correctors and potentiators, and combinations of these drugs will be tested in the screen. Proteostasis Therapeutics (PTI) is developing CFTR correctors, potentiators and an amplifier, which will all be tested in the drug screen. Drugs from FDL and PTI will be tested within the same screen because of their comparable modes-of-action. Eloxx Pharmaceuticals is developing a CFTR read-through drug that will be screened separately, since the distinct mode-of-action necessitates different assay conditions. Moreover, the drugs developed by FDL and PTI are not expected to work on premature termination codons. Therefore, only organoids with maximum one PTC will be screened for responsiveness to FDL and PTI drugs, and organoids with at least one PTC will be screened for efficacy of ELOXX drugs.

In both screens, organoids propagated in 24-wells plates will be transferred to 96-well plates for drug testing. Each experimental 96-wells plate will first go through several quality control checks to ensure that no pipetting errors have been made and the organoid cultures are of good quality by using reference organoid lines. These reference organoid lines are well characterized and the response of these reference organoids to the drugs from FDL, PTI and Eloxx should be within the range of what we have detected previously. CFTR function and response to the different drugs is measured by time-lapse confocal microscopy as the relative increase in surface area (as area under the curve, AUC) of calcein-green labelled organoids at 10 minute intervals for 60 minutes after stimulation with forskolin. Technological duplicates are measured for each condition, and individual drug responses are corrected for residual CFTR function upon forskolin stimulation. For FDL and PTI compounds, F508del homozygous organoids will be included in each experiment for comparison. For ELOXX compounds, G542X homozygous organoids will be included in each experiment for comparison. The best responding 27 organoids per lead product will be eligible for the clinical trials.

SNP fingerprinting analysis and *CFTR* mutation sequencing

As part of HUB’s quality control procedures, to confirm that no swap of material occurred during the whole screening process, genetic material from the biopsy tissue and the generated organoids will be isolated and submitted to SNP fingerprinting analysis. The obtained genetic material will be also used to confirm the *CFTR*

mutation by PCR-DNA sequencing of the patient derived organoids for the subsequent clinical trial.

Organoid biobanking for future use

The organoids generated will be stored by HUB for the duration of the study. During the HIT-CF project, a new biobank is being created to store the organoids for future use and make them available to researchers worldwide. Ethical experts will interview stakeholders and CF patients in order to explore the optimal governance of the biobank, and to generate an informed consent form that covers all aspects of this biobank.

Once this new biobank has been set up, patients will be asked to sign informed consent for permission to use their organoids in this biobank. This will only occur if the local study site has ethical approval for the separate biobank. If patients refuse, the organoids will be destroyed after the HIT-CF Organoid Study. If patients sign the informed consent, their organoids will enter the organoid biobank.

DISCUSSION

The HIT-CF Organoid Study applies intestinal organoids for personalized medicine in cystic fibrosis. Rare genetic variants of cystic fibrosis are currently excluded in CFTR-modulator trials, while it is expected that some of these variants are very responsive to treatment. By generating patient-specific organoids for these rare genotypes, a functional assessment of drug response per individual can be generated and treatment can be tailored to the individual. With the accompanying subsequent clinical trials, an innovative opportunity is created for CF-patients with rare mutations to get access to new CFTR-modulator drugs.

Personalized medicine approaches using organoids are ongoing in several diseases, but are still limited in scale. Several oncological trials are being conducted, such as in lung (clinicaltrials.gov identifier NCT03979170), breast (NCT03544047) and pancreatic cancer (NCT03544255). However, these studies are single-centre and aim to retrospectively compare *in vivo* and *in vitro* treatment responses. In the field of cystic fibrosis, pilot studies found that organoids can identify individuals that clinically benefit from the CFTR-modulating drugs ivacaftor and lumacaftor/ivacaftor with high accuracy, but these studies had a limited sample size [26,27]. By including up to 500 subjects in sites across Europe and performing confirmatory clinical trials, our project will be the first to prospectively assess the feasibility of using organoids for personalized medicine on such a large scale. One of the strengths of

this study is the collaboration with the European Cystic Fibrosis Society – Clinical Trials Network (ECFS-CTN). This network consists of 58 sites in 17 countries that share standardized working procedures and training, and have been involved in the study design. The study will be run only in ECFS-CTN sites, which will enable a quick start-up and ensures smooth communication between the sites and sponsor.

A risk of this project is that the drugs screened have yet to be clinically approved. It is therefore unclear whether all of these drugs will eventually reach the market and become publicly available. By involving three different pharmaceutical companies with several drugs in the pipeline and including ivacaftor and tezacaftor/ivacaftor in the drug screening, this risk is minimized. Another uncertainty is the number of responsive subjects that we expect to find. The current prediction of ~16% responsive subjects is based on a small dataset, and these numbers could differ depending on geographical distribution and efficacy of the experimental compounds. However, since drugs with innovative modes-of-action are investigated, the responsive population could be larger than previously anticipated. Moreover, efficacy of the experimental compounds is expected to be better than currently available drugs, which could result in a higher power in our clinical studies and therefore a reduced number of responsive subjects could be sufficient.

An important outcome of this study will be the large dataset created by combining all the individual results. This dataset will generate extensive knowledge on how different genotypes respond to drugs, and will become publicly available, which can be of great use to the scientific community. Furthermore, if the subjects consent, organoids that have been generated will be made available for future research. This biobank can be accessed by other scientists, and newly developed drugs can be tested on those organoids. Around 14 companies are currently developing drugs that aim to restore CFTR function (https://www.cff.org/Trials/pipeline) [35]. The impact of this study is therefore amplified, since many of these new drugs or experimental compounds could be screened.

The HIT-CF Organoid Study creates the opportunity of CFTR-modulating treatment in a CF patient population that has previously been largely ignored. Several drugs will be tested *in vitro* to stratify patients according to the best possible drug, thereby tailoring treatment to the individual patient. Moreover, extensive knowledge will be generated about the effects of drugs on a spectrum of genotypes, and the subsequently available biobank will be very useful for future drug development and research into Cystic Fibrosis.

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8

General discussion

GENERAL DISCUSSION

Therapies that improve CFTR-function can drastically alter the quality of life and life expectancy of people suffering from Cystic Fibrosis [1]. Nevertheless, currently available CFTR-modulators incur very high costs per patient and for the society as a whole [2,3] while treatment effects are highly heterogeneous. Therefore, it is essential to further optimize and personalize CFTR-modulator treatment. The current therapeutic landscape comprises a limited variety of CFTR-modulator therapies, which are all marketed by the same company. To increase cost-effectiveness of treatment these therapies need to be tailored to the individual, while generating alternative treatment options could decrease drug prices. This chapter discusses how organoids can (i) accelerate the preclinical development of drugs, (ii) optimize the use of currently available CFTR-modulators, (iii) increase access to CFTR-modulating therapy through personalized prediction of drug efficacy, and (iv) expand our knowledge of Cystic Fibrosis.

USING ORGANOIDS TO ACCELERATE PRE-CLINICAL DEVELOPMENT OF NEW CFTR-THERAPIES

The recent clinical success of CFTR-modulators has fortified the belief that drug development targeting dysfunctional CFTR is achievable, and the current pipeline of therapeutics in the pre-clinical and clinical phase contains more than 20 possible candidates with different modes-of-action (https://www.cff.org/Trials/Pipeline). Here, I highlight the current and potential use of organoids in accelerating the development of these therapies.

Rewriting the script- targeting the CFTR gene and CFTR-mRNA

Advances in genetic medicines have opened up new therapeutic avenues for CF, which is seen as a model monogenetic disease due to the well-defined disease pathophysiology. In addition, the phenotypic assessment of CFTR function (restoration) makes organoids a very useful model to test the feasibility of gene editing approaches. Techniques such as Crispr-CAS were successfully used to correct several CFTR-mutations in intestinal organoids [4,5], and these techniques have subsequently created helpful animal models that can accelerate compound testing in the late-preclinical phase [6,7].

Moreover, the large diversity of samples in our biobank has been used to study a recent addition to gene editing, namely adenine base editing. We showed that a significant fraction of biobanked organoids harbouring Premature Termination

Codons (PTCs) can potentially be rescued using this approach, while no off-target effects were detected [8]. These results are very promising, since base editing is an efficient and precise approach and could work for mutations that are not amenable to currently available CFTR-modulator treatment.

One of the major obstacles for gene therapy (i.e. inserting wild-type (WT) CFTR) is inefficiency of available vectors that transport the gene (or gene product) into the cell nucleus. Many trials have been conducted, and the only successful clinical trial could only detect a modest effect, most probably due to the use of a non-optimal vector and delivery method [9]. Intestinal organoids have been used to screen and validate different newly developed chimeric vectors [10], and have been important in the selection and validation of an rAAV vector that is currently being evaluated in a clinical trial for patients with CF [11].

Another very promising strategy is the use of antisense oligonucleotides (AONs) to correct mRNA defects including alternative exon splicing sites [12,13] that are not amenable to available CFTR-modulators. AONs are clinically available for several diseases including Spinal Muscular Atrophy [14], while an exciting new example of AON use has been in a patient suffering from Batten disease who had a unique mutation in the MFSD8 gene [15]. Using patient-derived cells an AON was specifically designed for this individual subject, and through expedited pre-clinical development, the patient received an effective drug within 1 year of discovering the genetic defect [15]. These results have sparked interest from many different research groups investigating a spectrum of diseases, and could be very interesting for further application in Cystic Fibrosis. As AONs need to be specifically designed for a mutation and validated pre-clinically before they can be introduced in the clinic, assessing whether the AONs improve CFTR function in organoids can be extremely helpful for rare mutations. The current HUB/UMCU biobank contains ~40 splice variants that could be targeted and could thus serve as a valuable resource for personalized AON development [8], while other biobanks might also contain unique splice mutations.

High-content drug screening to identify CFTR-modulating compounds

The high proliferation rate of intestinal tissue not only allows for long-term culturing, but also creates the opportunity for large scale high-content screening. Previously, libraries of G-protein Coupled Receptor (GCPR) compounds were screened in organoids, which identified beta-adrenergic agonists as activators of CF both in vitro and in vivo [16]. Since these drugs are clinically available and are generally safe, a clinical trial testing both oral and inhaled formulations was conducted, finding limited usefulness mainly due to side effects of the drugs, although in vivo CFTR-

function measurements suggested some improvement in CFTR-function [16] (G. Berkers, personal correspondence). High-content drug screening has been aided by the development of organoid screening in 384-wells plates in comparison to 96-wells, thereby significantly improving the throughput. This approach has been applied in the ongoing Rainbow project, where we aim to repurpose drugs for patients with rare mutations whose organoids are available in the biobank. By screening an FDA-approved drug library of >1400 compounds, the goal is to identify non-toxic, clinically available drugs that could improve CFTR function in individual patients. More than 180 organoids have been screened so far. Based on preliminary results, drugs appear not to restore CFTR function directly at a level of efficacy that is within the range of existing CFTR-modulators, but potentially beneficial drugs with different mode-of-action could help to enhance CFTR function or epithelial fluid transport. This could help to develop treatments for patients with rare mutations, who do not have treatment options thus far, and may lead to selections of drugs based on individual profiling.

OPTIMIZING CFTR-MODULATOR TREATMENT USING INTESTINAL ORGANOIDS

The last years have seen the remarkable introduction of CFTR-modulators for patients with CF. Ivacaftor, a CFTR-potentiator, entered clinical trials in 2007 and is very effective in improving lung function, BMI and quality of life in patients with gating mutations [23–25], while in vivo measurements of CFTR function also markedly improved [26,27]. However, CFTR-function and pulmonary function are not completely normalized through ivacaftor monotherapy, indicating the need for additional therapies. Moreover, ivacaftor did not lead to a clinical benefit in patients homozygous for the F508del mutation [28] which, in addition to gating defects, exhibits misfolding and aberrant trafficking of CFTR [29].

On top of potentiators, correctors were developed to enhance F508del CFTR protein folding. Combination treatment of ivacaftor and the first-generation correctors lumacaftor and tezacaftor results in improved lung function in patients with two F508del alleles, albeit with modest and highly variable effects between individuals [30–32]. Moreover, the treatment does not work sufficiently in F508del heterozygous patients, which suggests that F508del protein folding is not fully corrected [33]. In vitro experiments corroborate these clinical observations, since immature (B-band) CFTR is still detected in lumacaftor corrected F508del cells [34]. In silico predictions in combination with intestinal organoid testing previously indicated that next-generation correctors on top of lumacaftor could enhance the folding correction

[35]. Elexacaftor has been the first clinically approved next-generation corrector, and triple combination of tezacaftor/elexacaftor/ivacaftor treatment results in astonishing improvements for patients with at least one F508del allele, although large treatment effect variability can be observed [36,37]. A major issue with current CFTR-modulators are their extremely high prices that significantly limit access to these drugs for patients with CF, especially (but not solely) in less economically developed areas such as Eastern Europe. Even in the Netherlands and United Kingdom it has taken several years for lumacaftor/ivacaftor to be reimbursed [38,39].

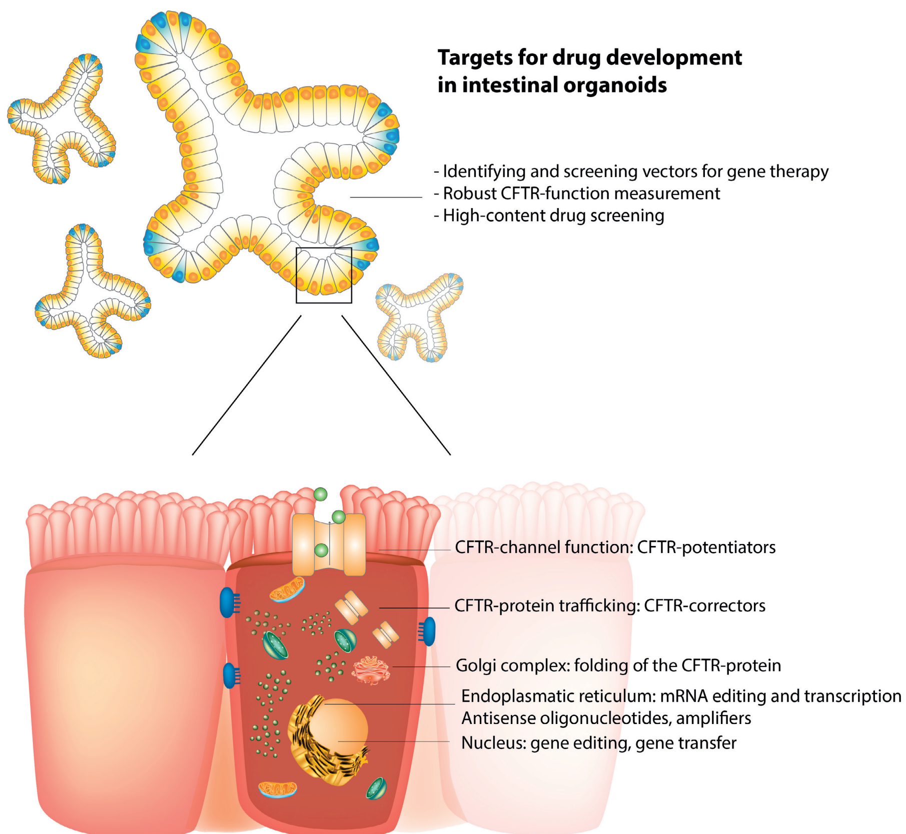

Figure 1: Different CFTR-targeting drugs can be researched using intestinal organoids. Intestinal organoids as a model can be very helpful to study the efficacy of potential gene therapy vectors, enable robust CFTR-function measurements and due to their high proliferation rate can be applied for high-content drug screening. Moreover, CFTR-modulating drugs that act on different cellular mechanisms can be studied.

Exploring CFTR-potentiator effects

The effect of CFTR-modulators on many *CFTR* variants other than F508del has not been thoroughly characterized, while it is known that some variants could be highly responsive [40]. Therefore, more research is needed to further study the effects of these drugs on (rare) *CFTR* variants. In this thesis, we explored the effects of CFTR-modulators on a range of *CFTR* variants. In **chapter 2**, we study organoids with the R117H allele in combination with a 7T poly-T tract and their response to ivacaftor [41]. A large variability in CFTR function was observed, consistent with literature on clinical presentations of individuals harbouring R117H-7T [42–45]. Interestingly, we found a correlation between CFTR-mRNA expression and ivacaftor response, with some organoids having low CFTR-mRNA expression and very limited responsiveness to ivacaftor. In addition, clinical trial data for patients harbouring R117H indicate large differences in response to treatment [46]. When combining these two observations, could it be that differences in mRNA expression explain response variability? So far, no data is available on whether mRNA expression could influence CFTR function and drug response of other mutations. If that should be the case, several interesting questions arise: could treatment be optimized by stratifying treatment according to in vivo CFTR-mRNA expression? Could patients with decreased mRNA expression especially benefit from additional treatment with an amplifier? These questions warrant further research into the origins of variability in CFTR-modulator response between individuals with similar genotypes, especially when regarding the high costs of these therapies and potential efficiency gains.

Since ivacaftor monotherapy does not normalize CFTR function in patients with gating mutations, **chapter 3** examines whether combination treatment of ivacaftor and the natural food supplements curcumin and genistein could boost CFTR-function of gating mutations [47]. All compound combinations showed a synergistic effect on organoid swelling as compared to monotherapy, most markedly for the combination of ivacaftor and genistein, which led us to trial these combinations in several subsequent clinical studies [48]. First, genistein/curcumin treatment was tested in a group of patients with the S1251N mutation, which did not result in a clear clinical benefit although an improvement in sweat chloride concentration was observed. Next, genistein was added on top of ivacaftor treatment, but did not lead to apparent improvements in clinical parameters. These somewhat disappointing results could be explained by the limited bioavailability of both genistein and curcumin [49,50], which is corroborated by the very low plasma concentrations of genistein and curcumin in patients participating in the trials. Further supporting this explanation is the fact that plasma from patients on active treatment with genistein and curcumin (which should thus contain these compounds) did not induce organoid swelling in vitro, while

plasma of patients on ivacaftor did result in organoid swelling [48]. Nevertheless, the study results imply that organoid swelling should not be the sole observation to drive clinical development, but ADME (absorption, distribution, metabolism and excretion) properties of drugs should also be taken into account so that experimental design of in vitro experiments are as predictive as possible.

The potential of elexacaftor

Elexacaftor is highly effective in combination with tezacaftor/ivacaftor [36,37], but limited pre-clinical information on it's mechanisms of action have been published [51]. Available literature suggests it works as a CFTR-corrector and improves folding and trafficking of F508del-CFTR to the plasma membrane. We are currently exploring the effects of elexacaftor on different CFTR alleles as monotherapy and in combination with tezacaftor and ivacaftor. Surprisingly, preliminary evidence suggests that elexacaftor acutely improves CFTR function in organoids with gating mutations in addition to its established F508del-CFTR correcting effects. Could elexacaftor thus be a dual corrector-potentiator, and could these characteristics explain the large improvements in in vivo CFTR function and lung function? Whether the potentiating effects of elexacaftor are additive or even synergistic to ivacaftor needs further study, but if this is the case, then the molecular target of elexacaftor might be different to ivacaftor. Further studies with e.g. patch-clamp recordings should elucidate the exact mechanisms of action of elexacaftor as a potentiator.

The studies described here indicate the potential of organoids to tailor treatment (combinations) and can help to improve our understanding of available therapies. With organoid biobanks, upcoming research questions can be efficiently studied using available tissues, thereby avoiding repeated and burdensome biopsies in patients. But can we also use the biobank for the direct benefit of the donors? i.e., is it possible to extrapolate organoid measurements to potential clinical benefit of individuals?

ORGANOID CFTR-FUNCTION MEASUREMENTS AS BIO-MARKER OF PATIENT-SPECIFIC OUTCOMES

In the next section, I will discuss if and how organoids could be used for personalized or precision medicine approaches. If organoids are to be implemented for this purpose, the first step in ensuring results used for individual predictions are reproducible is standardizing and harmonizing organoid culturing and assay conditions across laboratories. For this purpose, we collaborated with Hubrecht Organoid Technology and the CF research laboratories of the KU Leuven and University of Lisboa, and

published an open-access organoid culturing and FIS-assay protocol (**chapter 4)**. The protocol has been endorsed by the ECFS Standardization committee and the European Reference Networks for lung diseases, which will greatly help in the adoption of the protocol by other research laboratories. Moreover, to study whether results produced across laboratories are comparable, we have performed validation studies using a selection of six organoid lines distributed to each laboratory. Although small differences in swelling were observed between laboratories, agreement was generally high, and each separate organoid line behaved similar across laboratories (manuscript in preparation).

The observed differences highlight the feasibility of widespread implementation of this technology, but stress the need for experienced technicians and strict protocols, since the experiments were performed in laboratories with several years of experience in organoid culturing and FIS assays [4,52,53].

In **chapters 6 and 7**, we explore the relationship between organoid response to drugs and clinical benefit. **Chapter 6** explores improvements in several in vivo parameters in F508del homozygous patients treated with lumacaftor/ivacaftor and correlations with organoid measurements. No clear correlations could be identified on the individual level, although almost all patients showed improvement in at least one parameter and all organoids were responsive to lumacaftor/ivacaftor, albeit with variability in the extent of responsiveness. In **chapter 7** we studied the correlation between organoid response and clinical response to different CFTR-modulating compounds in patients with a broad range of genotypes, ranging from severe to mild mutations. Here, we found a strong correlation between organoid response and clinical response, and a large area under the Receiver Operations Characteristics curve, indicating high-to-excelled predictive value of organoids in this population [54]. How can we explain the discrepancy between the absent correlation in the F508del homozygous population and the high correlation in the wider range of genotypes?

Several mechanisms could have had an impact on these results. An important difference between the studies in chapters 7 and 8 is the choice of patient population. Chapter 7 explores differences between patients with identical CFTR-genotypes, while chapter 8 comprises patients with a wide range of genotypes but does include a significant proportion of patients with the S1251N mutation. Based on the results of chapter 8, two potential hypotheses could be generated: (i) organoids can be used to predict CFTR-modulator response on the individual level, and (ii) organoids can predict CFTR-modulator responsiveness of CFTR-genotypes, while chapter 7 seems to imply that prediction of individual responsiveness to lumacaftor/ivacaftor within the

F508del population is not possible. Nevertheless, we were able to find differences between individual organoids with identical genotypes in **chapter 2**, and a previous study into residual CFTR-function differences within the F508del homozygous population suggests that differences measured in organoids do correspond to clinical differences [55].

Another potential confounding factor is the study duration, which might have impacted on the ability to detect potential clinical improvements. While 8-16 weeks of lumacaftor/ivacaftor might not improve clinical outcomes such as $ppFEV_1$ or BMI, increased CFTR function was detected in all F508del homozygous patients using either FIS, Intestinal Current Measurement (ICM), Nasal Potential Difference (NPD) or Sweat Chloride Concentration (SCC). This suggests that more sensitive clinical outcome measures (i.e. endpoints that are able to detect small differences with more certainty), or longer clinical follow-up, might be necessary to unequivocally determine if individual patients benefit from lumacaftor/ivacaftor treatment. However, **chapter 7** mainly studies effects of ivacaftor with significantly better improvements in all clinical parameters in the responding population. The differences in results could possibly be explained by the intrinsic variability of the studied endpoints and biomarkers. ppFEV1 is the most commonly used endpoint in clinical trials, and is sensitive in detecting small average treatment effects in large populations, such as the average treatment response of ~3-4% to lumacaftor/ivacaftor in F508del homozygous subjects [31]. However, when it is used in a personalized setting, the measurement variability becomes problematic. Inter-measurement variability in stable subjects approximates 5% [56], which is larger than the expected treatment effect. Therefore, determining whether an observed change in ppFEV1 is due to a treatment effect or measurement variability is impossible, unless the change is far greater than could be accounted to variability. Many commonly used biomarkers in CFTR-modulator research such as sweat chloride concentration (SCC) and NPD show high inter-measurement variability [57,58], and therefore finding correlations is challenging if the estimated effect size is small.

Further impacting on the results found in these studies are factors that cannot be modeled in an in vitro system. Organoids harbor the unique genetic make-up of the individual donor, but are not exposed to environmental factors that impact on clinical disease severity, while environmental factors are thought to produce ~50% of pulmonary function variation [59]. These factors include, but are not limited to, air pollution [60], second-hand smoke exposure [61,62] and socio-economic status [63]. Since clinical disease severity could impact on drug responsiveness and experienced

side-effects causing increased discontinuation[64,65], these environmental factors could thereby impact on the correlations found between organoids and clinical effects.

In conclusion, current evidence suggests that organoids are able to detect clinically significant differences in CFTR-function and drug response between individuals, but that variability in short-term clinical endpoints between patients with identical genotypes reduce in vitro-in vivo correlations, especially in compounds with a modest effect size. Moreover, the exact impact of environmental factors on clinical drug response needs to be further clarified and taken into account when determining the value of organoids.

ORGANOIDS AS A TOOL FOR CLINICAL DECISION MAKING

So how could we use organoid response to drugs for clinical purposes? Different possibilities exist, including (i) identifying groups or individuals who could be further evaluated in clinical trials, (ii) as part of the drug label, allowing direct access to drugs for patients with a ‘positive’ organoid response, and (iii) to titrate the best treatment combination per individual once different CFTR-modulating therapies are clinically available.

Identifying novel populations for CFTR-modulating clinical trials

We have previously shown that organoid response clustered by genotype correlates very well to mean clinical response on group level [66]. If mutations can be identified that seem amenable to a certain drug based on organoid response, clinical trials can be designed in groups of patients with these mutations. This approached has been pioneered in a Dutch population harboring the A455E mutation (ClinicalTrials.gov Identifier: NCT03061331), and a clinical trial will also be performed in a multi-centre, international settting for patients with the R334W mutation (ClinicalTrials.gov Identifier: NCT04254705), and the 3849+10kb C>T and D1152H mutations (Clinicaltrials.gov Identifier: NCT03068312). These types of group-based trials need sufficient numbers of patients, which is impossible for ultra-rare mutations. For those patients with ultra-rare mutations, the HIT-CF (Human Individualized Treatment for CF) Europe project has been developed (www.hitcf.org). The first phase of HIT-CF is described in **chapter 8**, and involves generating organoids from 500 subjects with ultra-rare CFTR mutations who are currently devoid of CFTR-modulator treatment, and screening different CFTR-modulators for efficacy in patient-specific organoids (HIT-CF Organoid Study, Clinical Trial Identifier NTR7520). The best in vitro responding subjects will subsequently be asked to participate in clinical trials to

substantiate clinical benefit. Subject recruitment for the HIT-CF Organoid Study has been successfully completed with the inclusion of 502 subjects, and drug screening on their organoids is currently ongoing.

A risk of HIT-CF is whether the drugs included in the project will prove to be clinically effective and eventually reach market authorization. This is exemplified by the fact that that the project first included Galapagos NV and Flatley Discovery Labs, while these companies have had to upend their drug development for various reasons [67] (personal communication). Currently, Proteostasis Therapeutics and Eloxx pharmaceuticals are providing drugs for organoid screening and are fully committed to performing clinical trials in our population. The clinical trials are expected to enroll their first patients in the autumn of 2020, which will be a big step forward towards evaluating the value of organoids for precision medicine in CF.

Leaving no-one behind: organoids as a tool for access to CFTR-modulating drugs

The major goal of HIT-CF is to create access to CFTR-modulating therapy for patients that are currently devoid of treatment. How do these studies aid in reaching aforementioned objectives?

First, if the trials prove efficacy and safety of the drugs in our population, these studies will be part of the registration package of the drugs, allowing access for participants once market authorization and reimbursement of the drugs is approved. Secondly, successful trials will prove the feasibility and strength of using organoids to select individuals for clinical treatment. However, certain questions regarding organoid-based drug testing need to be answered before organoids can be implemented directly into clinical care, such as can we define swelling thresholds that predict clinical response versus non-response, and what are the positive and negative predictive values of these thresholds? With the available data from previous studies in combination with the newly generated HIT-CF data, we hope to elucidate some of these questions. Once valid thresholds are defined, organoids can be used to directly allow access to drugs on the basis of in vitro response, eliminating the need for clinical trials. This will allow young patients and patients with comorbidities who are excluded from clinical trials access to drugs. Especially in young children this approach could result in avoiding irreversible long-term damage.

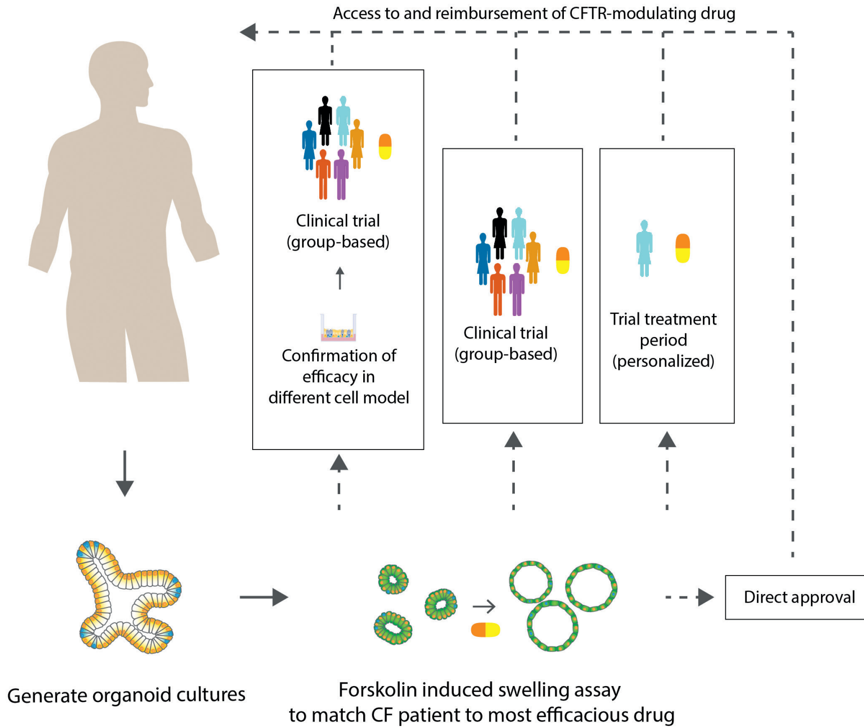

Figure 2. Different pathways for tailoring CFTR-modulating treatment using intestinal organoids

A plethora of companies are developing (combination) treatments that aim to enhance CFTR function. Due to the lack of effective therapy so far, clinical trials have been placebo-controlled. However, once CFTR-modulators are considered usual care, placebo-controlled trials are not ethical and new drugs should be compared against available options. These types of comparative or non-inferiority trials typically need large numbers of subjects to detect statistically significant differences, which is difficult in a rare disease such as Cystic Fibrosis with large heterogeneity in genotype. Moreover, certain mutations might be more amenable to specific modulators. So, how can we match the optimal treatment to each individual patient? Treating patients with different cocktails of drugs might be possible, but exposes patients to side effects of drugs, while the observed clinical differences might be insufficient to determine which treatment is superior. Here, organoids could potentially play an important role. We have previously shown that organoids can be used to compare effects of different CFTR-correctors and potentiators [54,66]. This approach could also be used to compare clinically available treatments in individual subjects, and could result in choosing the optimal treatment from a range of options based on organoid response.

Moreover, one could even imagine a scenario when e.g. the corrector from company X in combination with a potentiator from company Y would be superior to established combinations.

In order to efficiently use organoids for individualized therapeutic development, one first needs to generate and successfully culture the organoids and create universal access to these tissues. Once the tissue is widely available for research and clinical purposes, concerted international efforts could ensure that all (new) variants are studied and therapies are developed. Currently available biobanks are a first step towards that goal, but improvements could be made to further boost individualized medicine in CF.

THE ORGANOID BIOBANK AS AN INFINITE AND ACCESSIBLE RESOURCE FOR CF CLINICAL CARE AND RESEARCH

Intestinal organoids are obtained by isolating stem cells from rectal tissue, which can be safely and easily obtained through either forceps or rectal suction biopsy **(chapter 5)** [68,69]. Moreover, generating a large international biobank of organoids is feasible, which creates the opportunity to collect organoids with very rare mutations. Of the more than 2000 reported mutations, only 432 have been annotated, which leaves more than 1500 variants that have not been characterized (genet.sickkids.org, www.cftr2.org). These variants are extremely rare and sometimes unique for a single person or family, which complicates research into the pathogenicity and creates uncertainty for both the individual and the caregiver [52,70]. Furthermore, people with these variants are currently excluded from CFTR-modulator treatment since therapy is restricted to certain well-studied mutations included in the drug label [71,72].

In **chapter 5**, we show that with well-defined protocols, organoids can be generated from these individuals even if they live in a remote area and the cells thus have to travel long distances over extended periods. Outside of studies run in hospitals, determined individual patients have even managed to include their own organoids in the biobank by arranging biopsies and transport themselves [73].

Cystic Fibrosis organoids can be expanded, cultured and stored for prolonged periods while maintaining the CF phenotype [66], which enables the generation of a living biobank with (almost) infinite material for research purposes. Currently, a collaboration between Hubrecht Organoid Technology and the University Medical Centre Utrecht has resulted in a combined biobank of 664 CF organoid samples with

a large range of mutations that is accessible for researchers worldwide [8], while in parallel the HIT-CF Europe project will generate a biobank of 500 unique intestinal organoids of patients with two rare mutations **(chapters 5 and 8)**. Moreover, other research laboratories such as at the Katholieke Universiteit Leuven have set up similar biobanks for researching CF (K. de Boeck, personal correspondence).

While these biobanks are currently separate collections, their potential strength rests in their sample diversity, widespread accessibility and linkage to clinical data. This is especially true in CF, where even individual biobanks of more than 500 samples will not contain all the known CFTR variants. Therefore, creating combined databases of biobanks and large clinical datasets such as (inter)national patient registries would strongly increase the impact [74]. This approach is currently being pioneered in the Netherlands, where we are exploring the possibility of linking the organoid biobank to the Dutch Patient Registry. Moreover, combining existing organoid data to the CFTR2 database could aid knowledge on genotype-phenotype correlations. Future projects should focus on biobanking unique tissues and seek collaborations with existing biobanks/datasets to ensure maximum impact is thereby generated.

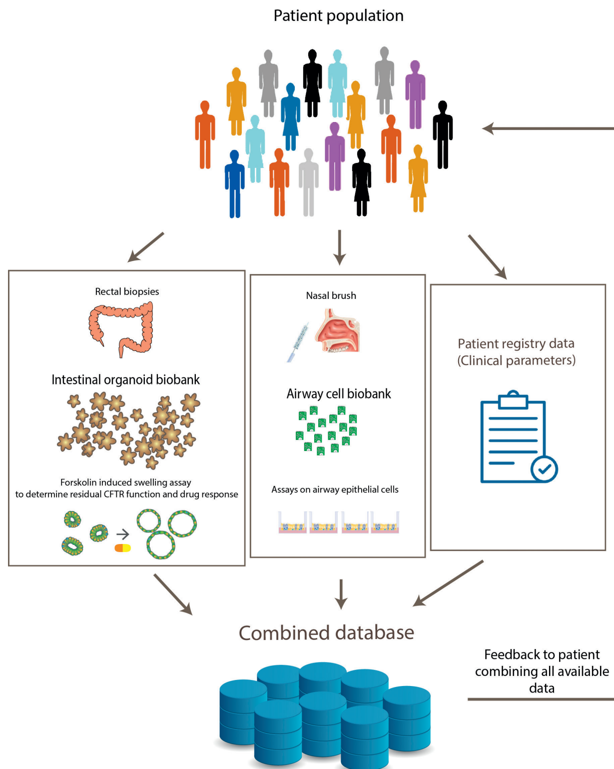

Figure 3. Combining biobanks and clinical data to support clinical care decisions

INTESTINAL ORGANOIDS IN PERSPECTIVE: COMPARISON TO OTHER MODELS

How do organoids compare to other ex vivo or in vitro cell models?

Cell-based approaches have mostly been used to type the average therapeutic response of a mutation or genotype. The Fisher Rat Thyroid (FRT) cell line in which CFTR mutations are introduced in a standardized manner using defined cDNAs can be used for high-throughput drug screening, and has helped to extend ivacaftor treatment to new CFTR mutations [75]. The strength of this model is its definition of standardized test components, and that patients do not have to undergo additional procedures. However, FRT cells are non-human, which influences CFTR-protein folding [76] and pharmacological treatment responsiveness [77,78]. Moreover, heterozygosity might influence CFTR-function, and these effects cannot be fully captured in this model. To complement this mutation-oriented approach, several models have been developed using patient-derived cells.

Only a few studies directly compare drug outcomes in ex vivo or in vitro cultured cells and in vivo parameters of treatment. Intestinal Current Measurement (ICM) on ex vivo rectal biopsies is validated for measuring residual CFTR function [79,80], and sensitively detects CFTR-modulator treatment effects after in vivo drug treatment [26,81]. In **chapter 6,** we found that both ICM on rectal biopsies and FIS in intestinal organoids could sensitively detect treatment effects of lumacaftor/ivacaftor on the F508del genotype, but the magnitude of response of the two biomarkers was not correlated. Thus, more research is needed to determine the role of and relationship between these biomarkers. The major downside of ICM is that only one therapy can be tested per biopsy, making personalized drug assessment burdensome, especially in an era with different treatment options.

CFTR function in Human Bronchial Epithelial (HBE) and Nasal Epithelial (HNE) cells is assessed using electrophysiological studies on air-liquid interface cultures, which mimics the airway environment [82]. High-throughput screening is not feasible due to cell senescence after a limited number of passages, and repeatedly obtaining HBE cells from living donors is an invasive process. HNE cells can be obtained by nasal brushing, and initial studies show that electrophysiological measurements in brushed HNE cells correlate with in vivo CFTR function and might be predictive of individual treatment response [83,84].

Compared to the abovementioned models, intestinal organoids have caveats. Because healthy organoids are pre-swollen, FIS cannot compare CF to healthy

control CFTR function. Moreover, although both intestinal and airway cells are derived from the endoderm, it is unclear whether intestinal epithelial CFTR function is directly related to airway epithelial CFTR function, which might influence treatment response [77,78]. Intestinal organoids are cultured as three-dimensional structures with the basolateral cell surface to the outside and the apical membrane facing the lumen of the organoid [17]. Thus, drugs added in vitro need to cross the basolateral membrane of the cell, which could impact absorption efficacy, especially for drugs that are designed to enter the cell via the apical membrane (such as inhaled drugs aimed at airway cells). Furthermore, the tissue most severely affected by CF is the airway epithelium, which differently expresses CFTR, differs with regards to cell turnover and protein homeostasis, and contains a range of other cell types than present in the intestinal epithelium [18–20]. Therefore, results cannot be directly extrapolated to airway cells. In contrast, airway epithelial cells can be used to study the mucus layer which is present in vivo and creates an additional barrier for compounds [21,22], and could thus provide useful information on the uptake of drugs and their effects on mucus (composition). A combination of primary cell models including AECs and two-dimensional cultures could therefore remain necessary to confidently assess potential of drugs for clinical application. Nevertheless, the potential to biobank and repeatedly test intestinal organoids facilitates robust personalized medicine approaches. Future studies are needed to elucidate which model is superior in terms of feasibility and predictive capacity.

CONCLUSION AND FUTURE DIRECTIONS

In this thesis, I describe the use of intestinal organoids for the development and tailoring of CFTR-modulating treatments. Available data indicate that intestinal organoids can be a very helpful tool to preclinically assess novel CFTR-modulating treatments and can serve as a useful model for developing new approaches such as gene editing. Moreover, currently available CFTR-modulator treatment can be repurposed and optimized through testing in intestinal organoids, while pilot studies indicate that we can predict clinical treatment effect in individual subjects using their intestinal organoids as a surrogate.

Although drug screening on organoids is currently possible in 384 wells format, this approach is time-consuming and expertise in organoid culturing is necessary. Moreover, data analysis has not been fully automated, limiting widespread implementation. In the future, screening platforms utilizing robots to culture organoids and artificial intelligence to analyse the data could increase the throughput and effectiveness of drug screening in organoids. In addition, studies directly comparing

intestinal organoids with other pre-clinical models such as HBEs and HNEs should further elucidate how CFTR-function in intestinal organoids relates to respiratory disease severity.

Future clinical studies should elucidate thresholds of organoid swelling that can predict whether a patient will or will not respond to a specific drug (combination), and whether these thresholds are identical for drugs with different modes-of-action, or whether drug-specific thresholds are necessary. Moreover, concerted efforts should ensure that widely available organoid biobanks are created that enable access to a diverse range of (intestinal) tissues to further expand our knowledge of CF and CFTR-directed therapeutics.

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Nederlandse samenvatting

HOOFDSTUK 1 – INTRODUCTIE

Cystic Fibrosis (CF) wordt veroorzaakt door mutaties in het '*Cystic Fibrosis Transmembrane Conductance Regulator*' (*CFTR*) gen. Deze mutaties leiden tot een verminderde of afwezige functie van het CFTR-eiwit. Het CFTR-eiwit is een ion-kanaal en transporteert chloride en bicarbonaat ionen aan de apicale zijde van met name epitheliale cellen. Dysfunctie van het CFTR-kanaal zorgt voor de typische ziekteverschijnselen van Cystic Fibrosis, zoals achteruitgang van longfunctie, longinfecties, alvleesklier-dysfunctie (o.a. diabetes) en darmverstoppingen. Het CFTR-kanaal is ook belangrijk voor het vloeistoftransport in de dikke darm, en is onder meer verantwoordelijk voor secretoire diarree zoals veroorzaakt door cholera.

Er bestaan meer dan 2000 verschillende mutaties in het CFTR-gen bekend, en deze mutaties kunnen elk een andere uitwerking hebben op de functie van het CFTR-kanaal. Sommige mutaties zorgen voor volledige afwezigheid van het CFTR-kanaal, terwijl andere ervoor kunnen zorgen dat het kanaal minder makkelijk open gaat en dus minder chloride kan transporteren. Van veel van deze mutaties is nog niet bekend wat voor uitwerking zij precies hebben op het CFTR-kanaal, terwijl de ernst van de mutatie veel invloed heeft op de ziekteverschijnselen van de patiënt. Als er meer bekend is over de uitwerking van verschillende mutaties kan een betere inschatting kan worden gemaakt hoe de ziekte per individu gaat verlopen. Dit kan onder andere in het laboratorium worden onderzocht in cellen afgenomen van mensen met CF.

Recente ontwikkelingen in de celbiologie hebben het mogelijk gemaakt om stamcellen te isoleren uit verschillende organen. Wanneer de stamcellen de juiste prikkels krijgen gaan ze zich vermenigvuldigen en zich driedimensionaal organiseren, waarbij ze gelijkenissen vertonen met het donororgaan. Deze driedimensionale celkweken worden daarom ook wel 'mini-organen' genoemd, ofwel 'organoïden'. Met deze mini-organen wordt onderzoek gedaan naar verschillende ziekten zoals kanker, ontstekingsziekten en aangeboren (genetische) ziekten zoals Cystic Fibrosis (CF).

Een van voorlopers van deze nieuwe technologie is de intestinale organoïd, het mini-darmpje. Door middel van een rectumzuigbiopt of een forcepsbiopt tijdens een colonoscopie, beiden procedures met een laag complicatierisico, kunnen stamcellen verkregen worden. Grote voordelen van intestinale organoïden zijn de mogelijkheid om ze vrijwel oneindig in leven te houden, snelle celdeling en het langdurig opslaan van de cellen in vloeibare stikstof voor toekomstig gebruik. Als

eenmaal intestinale organoïden beschikbaar zijn van een patiënt, kan hier vrijwel oneindig onderzoek mee gedaan worden. Met de intestinale organoïden doen we onder andere onderzoek naar nieuwe behandelingen voor Cystic Fibrosis.

Hoe intestinale organoiden helpen in het beter begrijpen van Cystic Fibrosis

In de intestinale organoiden van mensen met CF kunnen we de functie van het CFTR-kanaal meten. Door het activeren van het CFTR-kanaal met forskoline wordt zout en water naar het lumen van het mini-darmpje getransporteerd. Hoe beter de functie van het CFTR-kanaal, hoe meer water het mini-darmpje ingaat en dit veroorzaakt het opzwellen van het minidarmpje. De hoeveelheid zwelling is daardoor een indirecte maat voor de CFTR-functie. Dit noemen we de forskolin induced swelling (forskoline geïnduceerde zwelling, FIS) assay.

Mini-darmpjes van mensen met ernstige CFTR-mutaties zwellen helemaal niet, terwijl mini-darmpjes met mildere CFTR-mutaties (met verminderde, maar niet geheel afwezige functie van het CFTR-kanaal) wel zwelling laten zien. Verschillende studies hebben aangetoond dat de CFTR-functie gemeten in organoïden overeenkomt met de klinische ziekte-ernst van de patiënt en met andere biomarkers van CFTR-functie, zoals de chlorideconcentratie in het zweet. Bij mensen met zeldzame mutaties, waarvan niet bekend is wat voor effect deze hebben op de CFTR-functie, kan het helpen om hun mini-darmpjes te onderzoeken. Hierdoor kunnen we een idee krijgen over het effect van de mutatie op de CFTR-functie en dus ook de verwachte ziekte-ernst bij die individuele patiënt.

De FIS assay kan ook het effect meten van medicijnen die het CFTR-eiwit repareren, zogenaamde CFTR-modulatoren. Er zijn verschillende typen CFTR-modulatoren, waaronder CFTR-potentiators en CFTR-correctors. CFTR-potentiators binden aan het CFTR-kanaal waardoor deze makkelijker en langer open blijft staan, wat ervoor zorgt dat het ion-transport verbetert. Correctors zorgen ervoor dat het CFTR-eiwit in de cel beter gevouwen wordt, waardoor het minder wordt afgebroken in de cel en het een verbeterde functie heeft op het celmembraan. Deze medicijnen werken echter alleen voor bepaalde mutaties die hier gevoelig voor zijn.

Als intestinale organoiden met gevoelige CFTR-mutaties behandeld worden met CFTR-modulatoren, resulteert de verbeterde CFTR-functie in een grotere zwelling van het organoid. Door het vergelijken van onbehandelde en behandelde mini-darmpjes van dezelfde persoon, kan de toegevoegde waarde van een medicijn worden bestudeerd. Dit kan heel nuttig zijn voor patiënten met zeldzame mutaties, waarbij nog niet bekend is of er CFTR-modulatoren zijn die bij hen de CFTR-functie

kunnen verbeteren. In een eerdere studie hebben we twee CF-patiënten met zeldzame CFTR-mutaties geïdentificeerd bij wie de FIS assay een sterke verbetering liet zien na behandeling met ivacaftor (een potentiator), en hen vervolgens klinisch behandeld. Tijdens de behandeling gingen zij lichamelijk sterk vooruit, met een verbeterde longfunctie, verlaagd zweetchloride en verbeterde kwaliteit van leven. Dit zijn echter nog kleine studies, en meer onderzoek is nodig om de precieze rol en waarde van intestinale organoïden te bepalen.

Om de intestinale organoïden grootschalig te kunnen inzetten, moet meer bekend zijn over de voorspellende waarde van de FIS assay voor de klinische respons. Onderzoek met organoïden wordt inmiddels in meerdere laboratoria over de wereld gedaan. Daarom is het belangrijk dat alle laboratoria dezelfde manier van testen hanteren, zodat de resultaten vergelijkbaar zijn. In dit proefschrift onderzoek ik hoe we intestinale organoïden kunnen gebruiken voor de ontwikkeling van nieuwe medicijnen en gepersonaliseerde behandeling voor Cystic Fibrosis.

HOOFDSTUK 2 – HOEVEELHEID R117H-MRNA EN EIWIT EXPRESSIE ZORGT VOOR VERSCHIL IN CFTR-FUNCTIE

Een veelvoorkomende mutatie als oorzaak van CF is de R117H-CFTR mutatie. Cystic Fibrosis patiënten met de R117H mutatie tonen een grote variatie in ziekte-ernst. Een deel van de patiënten heeft een snelle achteruitgang van hun longfunctie, terwijl andere patiënten helemaal geen klachten hebben. Ook zijn er grote verschillen in het klinisch effect van ivacaftor in deze groep.

Het CFTR-gen (DNA) wordt in de cel vertaald in messenger RNA (mRNA). Dit mRNA wordt vervolgens gebruikt om het CFTR-eiwit te maken. Hoe meer mRNA er beschikbaar is, hoe meer eiwit er gemaakt kan worden (CFTR-kanaaltjes), en de hoeveelheid CFTR-kanalen is van invloed op hoeveel chloride-ionen er kunnen worden getransporteerd. Bij mensen met CF waarbij het CFTR-kanaal het minder goed doet, kunnen meer CFTR-kanalen ervoor zorgen dat er toch nog wat chloride-transport (restfunctie) is. Wij wilden onderzoeken of de hoeveelheid CFTR-mRNA bij patiënten misschien de oorzaak kan zijn van het verschil in ziekte-ernst.

In dit hoofdstuk vonden wij een sterke variatie in CFTR-functie in de intestinale organoïden van een groep van 14 patienten met de R117H-mutatie. Daarnaast bleek dat de CFTR-functie sterk gecorreleerd was met de CFTR-mRNA expressie en CFTR-ewit expressie. Ook vonden we dat het effect van ivacaftor op de CFTR-functie gecorreleerd was met de hoeveelheid CFTR-mRNA expressie. Deze bevindingen

suggereren dat de hoeveelheid CFTR-mRNA misschien gebruikt kan worden om de ziekte-ernst van individuele patiënten met een R117H mutatie te voorspellen, en mogelijk geldt dit ook voor patiënten met andere mutaties. Daarnaast kan de variatie in CFTR-mRNA expressie tussen patiënten mogelijk verklaren waarom er verschillen zijn in het klinisch effect van ivacaftor in deze groep.

HOOFDSTUK 3 – COMBINEREN VAN MEDICIJNEN EN VOEDINGSSUPPLEMENTEN OM CFTR-FUNCTIE TE VERBETEREN

Ivacaftor (een CFTR-potentatior) wordt voorgeschreven voor CF patiënten met de S1251N- en G551D-CFTR mutaties en zorgt voor een verbeterde longfunctie en kwaliteit van leven bij deze patiënten. Het zorgt ervoor dat de (niet goed werkende) CFTR-kanalen makkelijker open gaan en blijven, waardoor het chloride-transport toeneemt. De CFTR-functie van deze patienten is echter niet volledig genormaliseerd, en ivacaftor is erg duur. Uit eerdere onderzoeken is naar voren gekomen dat de voedingssuplementen genistein en curcumine ook CFTR-potentatiors zijn, en zouden een goedkoop alternatief kunnen zijn.

In deze studie bestudeerden we het gecombineerde effect van ivacaftor en de voedingssupplementen genistein en curcumine in intestinale organoïden met verschillende mutaties waaronder S1251N, G551D en F508del. Alle combinaties van deze medicijnen zorgden voor een synergistische verbetering van de CFTR-functie. In intestinale organoïden met de F508del mutatie die behandeld werden met zowel ivacaftor als lumacaftor, zorgden genistein en curcumine daarbovenop nog voor een verbetering in CFTR-functie. Dit suggereert dat het combineren van verschillende medicijnen een goede strategie kan zijn om de huidige klinische behandelingen voor CF te verbeteren.

HOOFDSTUK 4 – PROTOCOL VOOR HET GESTANDAARDISEERD KWEKEN VAN INTESTINALE ORGANOÏDEN EN HET METEN VAN DE CFTR-FUNCTIE.

Meerdere laboratoria wereldwijd gebruiken intestinale organoïden om onderzoek te doen naar Cystic Fibrosis. Die resultaten worden gebruikt om te beslissen of een persoon met CF misschien medicatie zou kunnen krijgen. Het is daarom erg belangrijk dat dit onderzoek zorgvuldig gebeurt, en dat resultaten tussen laboratoria vergelijkbaar zijn. Om deze resultaten te kunnen vergelijken, is het belangrijk dat iedereen op dezelfde manier de intestinale organoïden kweekt en CFTR-functie

meet. Voor dit doel hebben wij samengewerkt met de laboratoria van de KU Leuven, universiteit van Lissabon en Hubrecht Organoid Technology om onze kweek- en meetprotocollen te standaardiseren. Het definitieve protocol legt in detail uit welke materialen er nodig zijn, hoe je kwalitatief goede resultaten krijgt van de FIS assay en hoe deze te verwerken.

HOOFDSTUK 5 – HOE VEILIG EN EFFICIENT IS HET VERKRIJGEN VAN MINIDARMPJES?

Intestinale organoïden worden in toenemende mate gebruikt voor onderzoek naar darmkanker, ontstekingsziekten zoals de ziekte van Crohn en erfelijke ziekten. Om grote internationale studies op te zetten, is het daarom belangrijk om te weten hoe veilig het is om darmbiopten af te nemen. Hoeveel last heeft de patiënt, en wat is het risico op complicaties? Daarnaast is het nuttig om te weten wat het succespercentage is van het verkrijgen van intestinale organoïden uit deze biopten. Als dit proces vaak mislukt, kan dit ervoor zorgen dat studies niet slagen, of dat patiënten keer op keer terug moeten komen naar het ziekenhuis voor een nieuw biopt.

In de HIT-CF Organoid Study zijn van 502 personen met CF uit 33 verschillende ziekenhuizen en 12 landen biopten afgenomen om intestinale organoïden te kweken. Beide procedures hadden een laag complicatierisico van minder dan 1 %, met in totaal twee serieuze complicaties (een bloeding en een gastro-enteritis). Daarnaast was de slagingskans voor het verkrijgen van organoïden 95 %. Hieruit blijkt dat het veilig en goed mogelijk is om internationale onderzoeken op te zetten die gebruikmaken van intestinale organoïden.

HOOFDSTUK 6 – KUNNEN WE DE KLINISCHE EFFECTEN VAN LUMACAFTOR/IVACAFTOR VOORSPELLEN BIJ PATIENTEN MET DE F508DEL MUTATIE?

Lumacaftor/ivacaftor is een geregistreerde behandeling voor patiënten met Cystic Fibrosis veroorzaakt door twee kopieën van de F508del mutatie in het CFTR-gen. Echter is er een grote variabiliteit in klinisch effect tussen de patiënten. Ook de FIS assay laat een grote variabiliteit zien in effect van lumacaftor/ivacaftor tussen intestinale organoïden van patiënten met de F508del mutatie. Lumacaftor/ivacaftor is een duur medicijn en een deel van de patiënten heeft (ernstige) bijwerkingen. Het zou daarom nuttig zijn om te kunnen bepalen welke patiënten wel en geen baat hebben van deze behandeling. Er zijn verschillende manieren om een effect van behandeling te meten. Dit kan te merken zijn doordat de longfunctie is verbeterd, maar ook

door de activiteit van het CFTR kanaal in het lichaam te meten door middel van intestinale stroommetingen, neuspotentiaalmetingen en de chloride concentratie in het zweet (biomarkers). In deze studie wilden wij onderzoeken of de CFTR-activiteit in intestinale organoiden gerelateerd is aan de verbetering in CFTR-functie bij een van deze metingen na behandeling met lumacaftor/ivacaftor.

In een groep van 21 F508del homozygote patiënten met CF hebben wij onderzocht welke biomarkers en klinische uitkomstmaten een behandeleffect lieten zien van lumacaftor/ivacaftor, en of deze effecten onderling gecorreleerd waren. We vonden in de intestinale organoïden van alle patiënten een effect van lumacaftor/ivacaftor, maar er was een groot verschil in effect tussen patiënten zichtbaar. Deze variabiliteit was in onze studie echter niet gecorreleerd met de andere biomarkers van CFTR-functie. Ook was de mate van zwelling in de intestinale organoïden in deze groep niet gecorreleerd met verbetering in longfunctie. Onderling was er ook geen correlatie tussen de verschillende biomarkers en klinische uitkomstmaten. In deze studie is wel te zien dat alle intestinale organoïden een respons laten zien op lumacaftor/ivacaftor, terwijl dit bij andere metingen soms niet opgepikt kon worden. Bij enkele patiënten was een duidelijk effect meetbaar in zweetchloride, terwijl hun longfunctie niet verbeterde. In de praktijk blijft het dus erg lastig om te bepalen bij welke patiënt het medicijn effect heeft. Het lijkt daarom belangrijk om meerdere biomarkers te meten, om het mogelijke effect van een medicijn goed te kunnen beoordelen.

HOOFDSTUK 7 – GEPERSONALISEERDE BEHANDELING VOOR CYSTIC FIBROSIS

Er zijn meer dan 2000 mutaties in het CFTR-gen bekend die Cystic Fibrosis kunnen veroorzaken. Een deel van die mutaties zijn te behandelen met CFTR-modulatoren zoals ivacaftor en lumacaftor, maar het is op basis van de locatie van de mutatie in het CFTR-gen alleen niet goed te voorspellen welke mutaties dit zijn.

In deze studie hebben we gekeken hoe goed de intestinale organoïden het klinisch effect van deze medicijnen kan voorspellen bij mensen met verschillende CFTR-mutaties. Meer dan 20 patiënten met verschillende CFTR-mutaties werden behandeld met (een combinatie van) ivacaftor, lumacaftor, curcumine en genistein. Daarnaast werd het effect van die medicijnen gemeten door middel van de FIS assay in intestinale organoïden. De FIS assay bleek te kunnen voorspellen welke patienten een verbetering in zweetchloride of longfunctie zouden laten zien, met een hoge sensitiviteit en specificiteit. Ook patiënten met zeldzame mutaties, waarvan nog niet bekend was dat deze te behandelen waren met medicijnen, reageerden

sterk op de behandeling. Het lijkt dus mogelijk om op basis van de FIS assay te voorspellen welke patiënten met zeldzame mutaties wel en niet reageren op CFTR-modulerende medicijnen.

HOOFDSTUK 8 – DE HIT-CF ORGANOID STUDY

CFTR-modulatoren zijn beschikbaar voor patiënten met 34 verschillende CFTR-mutaties, terwijl er meer dan 2000 bekend zijn. Dit betekent dat veel van patiënten met zeldzame CFTR mutaties niet worden behandeld met CFTR-modulatoren, terwijl ze daar misschien wel baat bij kunnen hebben. Voortbordurend op de resultaten uit hoofdstuk 7 worden in de HIT-CF Organoid Study intestinale organoïden gekweekt van meer dan 500 patiënten uit Europa die een zeer zeldzame CFTR-mutatie hebben. Op deze intestinale organoïden worden nieuw ontwikkelde CFTR-modulatoren getest van verschillende biotechnologie en farmaceutische bedrijven, om te kijken of we patiënten kunnen vinden die goed kunnen reageren op die medicijnen. Uiteindelijk worden de patiënten wiens intestinale organoïden het best reageren, uitgenodigd om mee te doen aan een klinische studie, waarin we het effect van de medicijnen op de patiënt zelf willen onderzoeken. Hiermee hopen we effectieve medicijnen te vinden voor deze achtergebleven patiëntengroep, en meer informatie verzamelen over de voorspellende waarde van de intestinale organoïden.

CONCLUSIE

In dit proefschrift hebben we onderzocht hoe intestinale organoïden kunnen helpen met het beter toepassen van CFTR-modulatoren. Het verkrijgen van organoïden door middel van darmbiopten is een haalbare, veilige en effectieve manier om internationale studies en biobanken op te zetten. Intestinale organoïden kunnen helpen om variatie tussen patiënten met hetzelfde genotype te onderzoeken en gebruikt worden om bestaande medicijnen of voedingssupplementen te herpositioneren voor Cystic Fibrosis. Of we binnen een groep patiënten met hetzelfde genotype kunnen bepalen wie wel en niet op medicijnen reageert moet verder worden uitgezocht. Wel kunnen mensen met zeldzame CFTR-mutaties geïdentificeerd worden die baat hebben bij klinische behandeling met een CFTR-modulator. Dit wordt grootschalig verder onderzocht in het Europese HIT-CF project. Met intestinale organoïden kunnen we hopelijk in de toekomst voor iedere patiënt met CF zorgen voor gepersonaliseerde behandeling.

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LIST OF ABBREVIATIONS

Ad-DF+++	- Advanced Dulbecco's Modified Eagles Medium with Nutrient Mixture F-12 Hams (Ad-DF) 500ml +1 % glutamax/1 % hepes buffer/1 % pen-strep)
AON	- Antisense oligonucleotides
ATP	- Adenosinetriphosphate
AUC	- Area under the curve
BMI	- Body mass index
cAMP	- Cyclic adenosine monophosphate
CF	- Cystic Fibrosis
CFTR	- Cystic Fibrosis Transmembrane Conductance Regulator
CM	- Colon organoid medium
DMEM	- Dulbecco's Modified Eagles Medium
DMSO	- Dimethyl sulfoxide
ECFS-CTN	- European Cystic Fibrosis Society – Clinical Trial Network
EDTA	- Ethylenediaminetetraacetic acid
ELOXX	- Eloxx Pharmaceuticals
EMA	- European Medicines Agency
FBS	- Fetal bovine serum
FDL	- Flatley Discovery Labs
FDA	- Food and Drug Administration
FIS	- Forskolin induced swelling
FRT	- Fisher rat thyroid
g	- Gravity (units)
GCPR	- G-protein coupled receptor
HBE	- Human bronchial epithelial
hEGF	- Human epithelial growth factor
HIT-CF	- Human Individualized Treatment for CF
HNE	- Human nasal epithelial
HUB	- Hubrecht Organoid Technology
HSP90	- Endogenous heat shock protein 90
ICM	- Intestinal Current Measurement
IRB	- Institutional Review Board
IQR	- Interquartile range

KUL	- Katholieke Universiteit Leuven
LIS	- LIS
μ-/m-/ l	- micro-/ milli-/ liter
μ-/m-/M	- micro-/ milli-/ molar
Nac	- N-acetylcysteine
NCM	- Noggin conditioned medium
NPD	- Nasal Potential Difference
PBS0	- Phosphate Buffered Saline (without magnesium and calcium)
$ppFEV_1$	- Percentage predicted forced expiratory volume in one second
RhoKi	- Rho- Kinase (ROCK inhibitor)
ROC	- Receiver operation characteristic
R-spo3	- hR-spondin-3
RT	- Room temperature
SD	- Standard deviation
SLA	- Steady state lumen area
SCC	- Sweat Chloride Concentration
Poly-T	- Poly-thymidine
PTC	- Premature termination codon
PTI	- Proteostasis Therapeutics
UMCU	- University Medical Centre Utrecht
WCM	- Wnt-3A conditioned medium
WT	- Wild type

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CURRICULUM VITAE

Peter van Mourik was born on March 6, 1990 in IJsselstein, the Netherlands, where he lived throughout his childhood. After graduation at the Cals College in Nieuwegein in 2008, he started his medical training at Utrecht University. He did international internships at the Himalaya Eye Hospital, Pokhara, Nepal, and the University of Cape Town, South Africa. In 2013 he did a 6-month scientific research project in the lab of prof. dr. J.M. Beekman, which sparked his interest in Cystic Fibrosis research. In 2014 he obtained his medical degree and at that same year he started working as a resident for internal medicine, pulmonology, cardiology and gastroenterology at Ziekenhuis Gelderse Vallei, Ede.

In 2016 he started research projects for his PhD trajectory at the University Medical Center Utrecht University Medical Center Utrecht under supervision of prof. dr. C.K. van der Ent and prof. dr. J.M. Beekman at the department of pediatric pulmonology. In 2018, he moved to Perth, Australia for one year of research at the Telethon Kids Institute under supervision of prof. dr. Steven Stick and dr. Anthony Kicic. In 2020 Peter started working as pulmonology resident at the University Medical Center Utrecht, under supervision of prof. dr. H. Heijerman and dr. R.C. Schweizer. Peter is married to Maxime Molenaar, and together they have one son: Marien (2020)