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Early View

Original article

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Stratifying infants with cystic fibrosis for disease severity using intestinal organoid swelling as biomarker of CFTR function

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Take home message for social media:
'Laboratory-grown mini-guts inform on individual disease characteristics of infants with cystic fibrosis'

Abstract

Forskolin-induced swelling (FIS) of intestinal organoids from individuals with cystic fibrosis measures function of the cystic fibrosis transmembrane conductance regulator (CFTR), the protein mutated in cystic fibrosis (CF).

We investigated whether FIS corresponds with clinical outcome parameters and biomarkers of CFTR function in 34 infants diagnosed with CF. Relations between FIS were studied for indicators of pulmonary and gastro-intestinal disease.

Children with low FIS had higher levels of immunoreactive trypsinogen (IRT, $p=0.030$) and pancreas associated protein ($p=0.039$), were more often pancreatic insufficient (PI, $p<0.001$), had more abnormalities at chest computed tomography ($p=0.049$), and lower z-scores for maximal expiratory flow at functional residual capacity ($p=0.033$) when compared to children with high FIS values. FIS significantly correlated with sweat chloride concentration (SCC) and intestinal current measurement (ICM), $r = -0.82$ and 0.70, respectively, both $p<0.001$. Individual assessment of SCC, ICM and FIS suggested that FIS can help to classify individual disease severity.

Thus, stratification by FIS identified subgroups that differed in pulmonary and gastrointestinal outcome parameters. FIS of intestinal organoids correlated well with established CFTR-dependent biomarkers such as SCC and ICM, and performed adequately at group and individual level in this proof-of-concept study.

Introduction

In vitro tests using cultures of sustainable living patient tissues from biobanks might provide a patient-friendly and cost-effective alternative for in vivo testing in clinical care settings. Our identification of LGR5 as stem cell marker led to the establishment of stem cell based organoid cultures and the storage of such tissue in living biobanks [1–4]. The value of these resources for individual clinical care remains unclear as direct studies comparing in vitro results from such stem cell-derived cultures with individual clinical characteristics are lacking.

We here studied whether intestinal organoid cultures could be used to inform on individual disease characteristics of people with cystic fibrosis (CF), a monogenetic life-shortening rare disease caused by mutations of the *Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)* gene [5–7]. Intestinal organoids are in vitro-cultured multicellular, three-dimensional epithelial structures that mimic the in vivo intestinal epithelium including stem cell self-renewal, multilineage differentiation, and cell polarity, and can be stored in a living biobank [2,8]. We recently developed an assay using intestinal organoids to quantitate CFTR function [9]. The CFTR protein functions as an anion channel at many mucosal surfaces and is a critical regulator of ion and fluid homeostasis [10]. CFTR activation by forskolin induces luminal fluid secretion and rapid swelling of organoids, which is absent or strongly reduced in organoids from subjects with CF. Current data indicate that forskolin-induced swelling (FIS) is a CFTR-dependent readout and enables a fast, robust and precise typing of CFTR residual function in vitro as demonstrated previously [9,11].

CF is a progressive multi-organ dysfunction characterized by accumulation of viscous mucus in the pulmonary and gastrointestinal tract resulting in bacterial infections, chronic inflammation and malnutrition. A high degree of variability in organ dysfunction and survival exists between subjects with cystic fibrosis, which can be caused by variation in the *CFTR* gene itself as over 2000 *CFTR* variants have been reported (www.genet.sickkids.on.ca), as well as additional genetic and environmental factors [12]. Hence, clinicians face great difficulties to predict the clinical course of the individual patient based on the CFTR genotype, especially for subjects carrying rare CFTR variants (www.CFTR2.org) [13].

Individual biomarkers of CFTR function play an important role for diagnosing CF or other CFTR-related diseases [14]. In vivo sweat chloride concentration measurements (SCC), and intestinal current measurement (ICM) on ex vivo rectal biopsies are established diagnostic biomarkers of CFTR function. These biomarkers have been validated in clinical studies which has led to thresholds for CF diagnosis and disease severity classification. These studies demonstrated that residual CFTR function associates with *CFTR* genotype and disease severity at group level [13,15–25]. However, these biomarkers are also associated with considerable technical and non-CFTR dependent biological variability, which may limit their capacity to precisely assess individual CFTR function and consequently may lower their capacity to individually inform on disease severity [20,26–28].

FIS offers a sensitive and precise determination of individual CFTR function but also a culture-dependent bias, and thus far has never been assessed in the context of clinical CF disease presentation. The aim of this study was to define relations between FIS in intestinal organoids

and clinical outcome parameters in a cohort of consecutive newly diagnosed infants with CF. We also compared how FIS of organoids correlated with SCC and ICM and how they inform on clinical outcome parameters at the individual level.

Materials and methods

Infants with CF identified by newborn screening and treated by the CF clinics of the University Medical Center Utrecht or the Erasmus Medical Center Rotterdam enrolled in a standardized monitoring protocol, adapted from the AREST-CF protocol [29,30]. The Dutch protocol for newborn screening for CF includes measurement of Immunoreactive Trypsinogen (IRT) and Pancreatic Associated Protein (PAP) in heel prick blood [31]. The monitoring protocol includes a sweat chloride concentration (SCC) test and CFTR-genotyping after birth and bacterial airway cultures at every regular outpatient visit. At one year of age, patients underwent general anesthesia for chest CT scanning, bronchoscopy with collection of bronchoalveolar lavage (BAL) fluid and rectal suction biopsies for ICM and organoid cultures. Patients from Rotterdam also performed infant lung function testing (ILFT). See supplementary materials for a detailed description of the monitoring protocol (S1). The ethics committees of the University Medical Center Utrecht and Erasmus Medical Center Rotterdam approved use of rest material of rectal biopsies for culture of organoids and use of clinical data. Informed consent was obtained from all parents and caregivers of participating subjects.

Measurements of SCC were performed according to the standard operating procedure of European Cystic Fibrosis Society-Clinical Trial Network (ECFS-CTN), both Utrecht and Rotterdam

are certified centers to perform sweat tests [18,32]. ILFT, chest CT, bronchoscopy, and BAL were performed according to standardized procedures [33–35]. For ILFT measurements of forced expiration using rapid thoraco-abdominal compression (RTC) techniques and the forced deflation technique were used. See online supplements (S2) for CT procedures. Severity of airway disease (% disease) was scored in random order, blinded to patient identifiers using the PRAGMA-CF CT scoring method. % Disease reflects the percentage of total lung volume showing bronchiectasis, bronchial thickening, or mucus plugging [36]. Bronchoscopy was performed under general anesthesia. BAL was done with 3 aliquots of 1 ml/kg of NaCl 0.9% each in the right middle lobe and 1 aliquot in the most affected lobe. Intestinal current measurements in rectal biopsies were performed using a standardized protocol [19,37], see supplementary materials for a detailed description (S3). The cumulative response to carbachol, forskolin, and histamine was used for analyses.

Methods for generation of intestinal organoids and measurement of forskolin-induced swelling were slightly adapted from protocols described previously [9,11,38]. Additional detail on these methods is provided in an online data supplement (S4).

Different analysts executed sweat tests, ICMs and FIS assays, and analysts were blinded for the outcome of other biomarkers of CFTR function and in vivo observations.

Clinical data were collected from the patients files retrospectively from the first year of life from the moment of diagnosis by newborn screening.

Statistical analysis

First, descriptive statistics of clinical parameters were used to describe the study population. Then we evaluated whether the study population could be divided into distinct subpopulations based on FIS results across forskolin concentrations. For this, FIS results were transformed to obtain standard normal distributions, and then agglomerative hierarchical Ward clustering with Euclidean distance was used. We determined the number of clusters apparently present in the data based on a majority vote of 30 different cluster indices [39], and then assessed whether FIS results at a single forskolin concentration could identify the resulting FIS patient clusters.

We then compared clinical parameters between the identified FIS patient clusters, reporting medians and interquartile range and performing Mann-Whitney-U tests for non-normally distributed continuous data, means and standard deviation and performing t-tests for normally distributed continuous data, and numbers and percentages with Fisher's exact tests for categorical data. Similarly, clinical parameters were compared between patient groups according to SCC and established thresholds of this biomarker.

We also directly compared the continuous FIS results with the continuous SCC and ICM results using scatterplots, estimating the Pearson correlation coefficient, and using linear regression.

R 3.2.1 for Mac was used for all analyses and p-values were reported based on two-sided tests.

Results

Between May 2011 to January 2015 34 newborns were enrolled. Table 1 shows clinical characteristics of the study population during the first year of life. ICMs were available from subjects from Rotterdam (n=23), but could not be determined for patients from Utrecht for technical reasons. Values of all available and technically reliable ICM responses are shown in an online supplemental table (S5).

Forskolin dose-dependently induced swelling of organoids in a patient and CFTR-genotype-dependent fashion (Fig. 1a,b). In total, we measured 1552 datapoints, and censored 9 points as these were qualified as extreme outliers (> 6SD difference from the average of the experimental replicates). Only forskolin concentrations of 0.128 µM or more induced discernible organoid swelling, which was largest at 5 µM. A cluster analysis based on FIS values of all forskolin concentrations from 0.128 to 5 µM robustly identified two different groups (FIS-high n=9, FIS-low n=25, Fig. 1c). These two groups could also be accurately identified in figure 1a at forskolin concentrations of 0.8 µM or higher with an AUC threshold of 1000.

Clinical parameters were compared between groups with high versus low FIS (Table 2). Subjects with low FIS (FIS < 1000 AUC at 0.8 µM forskolin) compared to subjects with high FIS had higher IRT (160 vs 123 µg/ml, p=0.030) and PAP concentrations (5.9 vs 3.0 µg/ml, p=0.039), were more frequently pancreatic insufficient (19/25 vs 2/9 patients, p<0.001), had higher PRAGMA-CF CT scores for % disease (3.6 vs 1.8, p=0.049) and lower z-scores for maximal expiratory flow at

functional residual capacity (Vmax-FRC) (-1.9 vs -0.2, p=0.033). These data demonstrate that FIS is an important indicator of relevant clinical parameters during the first year of life.

At 0.8 µM forskolin, FIS of organoids from all individual patients was significantly correlated with paired *in vivo* SCC (r= -0.82 (95%CI -0.91- -0.68, p=1.97e^{-9}, Fig. 2a) and with *ex vivo* ICM (r= 0.70 (95%CI 0.41-0.86, p=1,93e^{-4}, Fig. 2b). Concluding, FIS values significantly correlate with current clinically established *in vivo* and *ex vivo* CFTR-dependent biomarkers.

We next compared clinical parameters between groups with high versus low values of FIS and SCC (Table 2). We did not compare ICM values with clinical parameters as the significant lower observations may introduce a biased interpretation. To divide groups with high and low SCC values for severe or milder phenotypes of CF, we used the generally accepted SCC borderline of 60 mmol/L [18,40,41]. Patients with high SCC values (> 60 mmol/L) compared to patients with low SCC values had higher IRT concentrations (160 vs 109 µg/ml, p=0.012), were more frequently pancreatic insufficient (20/26 vs 1/8 patients, p<0.001), and had higher PRAGMA-CF CT scores for % disease (3.7 vs 1.3, p=0.007). The subgroups identified by high or low FIS or SCC values differed by three individuals (nr. 13, 26 and 27) in this cohort. In conclusion, FIS-based subgroups associated with 5 out of 9 studied clinical endpoints during the first year of life in comparison to 3 for SCC-based subgroups.

The above group-based associations with clinical disease severity suggest that FIS may have additional value for interpreting individual clinical disease. We compared individual measures of FIS, SCC and ICM, and borders to discriminate between low or high residual CFTR function (for FIS), or mild or severe CF phenotype for SCC (60 mmol/L) and ICM using the 10 $\mu A/cm^2$ as threshold for ICM [19]. FIS shows a clear dichotomous distribution of data (Fig. 3a), in contrast to more gradually dispersed values of SCC and ICM (Fig. 3b,c). In general, low FIS values corresponded with SCC beyond 60 mmol/L and ICM values below 10 $\mu A/cm^2$, but some discrepancies were observed. For example, individual nr. 4 with a clinical severe phenotype (pancreatic insufficiency and need for antibiotic prophylaxis before the age of 6 months), showed low FIS and correspondingly high SCC values, but unexpectedly a very high ICM value. One individual with a very low FIS (nr. 13) was typed with an intermediate SCC and low ICM. Clinical data for this individual suggested a severe disease phenotype based on the CFTR genotype (CFTR2), pancreatic insufficiency (fecal elastase < 15 µg/g) and need for 8 antibiotic treatments because of pulmonary symptoms in the first year of life. Two other individuals having SCC > 60 mmol/L (nr. 26 and 27) showed high FIS levels (ICM was also high for nr. 27 and not available for nr. 26). These subjects also displayed milder phenotype as illustrated by pancreatic sufficiency (fecal elastase > 500 µg/g) in both individuals and hardly any pulmonary symptoms. In conclusion, the data suggest that FIS may have added value to assess individual disease characteristics next to SCC and ICM.

Discussion

This study investigated the performance of an in vitro biomarker in cultured adult stem cells for identifying clinically distinct subgroups in 1-year-old children with cystic fibrosis. We found that stratification by FIS of intestinal organoids identified subgroups that differed in pulmonary and gastrointestinal clinical outcome parameters. FIS correlated well with currently established CFTR-dependent biomarkers as SCC and ICM, and performed adequately at group and individual level to inform on relevant pulmonary and gastrointestinal disease phenotypes. The FIS measure integrates the impact of both CFTR mutations and other patient-specific modifier genes expressed in intestinal cell cultures that act upon CFTR function. It is likely that a strong dependency of FIS on CFTR, as well as optimal sensitivity and precision due to repeated measurements enables an accurate individual estimation of *in vivo* CFTR residual function, thereby facilitating linkage to disease characteristics.

Our cluster analysis based on FIS values of forskolin concentrations beyond 0.128 µM convincingly divided this consecutive group of infant CF patients into two separate groups. FIS at 0.8 µM forskolin precisely identifies the two above-described clusters, and it therefore seems sufficient to measure FIS values at less forskolin concentrations for typing CFTR residual function. Impressively, the cluster analysis of FIS data of only a small amount of subjects yielded distinct subgroups that were clearly associated with clinical disease severity indicators, essentially yielding similar data when the cohort was divided into subgroups using SCC-based criteria previously established in larger studies [40,41].

The most important finding in this study demonstrated that CFTR function as measured by FIS points out to be an indicator of relevant clinical parameters (Table 2). CF disease is driven by

CFTR mutations, other modifier genes and environmental factors, and the impact of these variables change in relation to specific disease manifestations and age. Whereas CFTR function is the primary determinant of pancreatic exocrine dysfunction [12], and CFTR function measurements will be highly informative, variation in airway obstruction and pulmonary infection are strongly modified by other genetic modifiers and environmental factors [12]. By focusing on early disease characteristics in young infants, we might have studied clinical phenotypes that are more directly related to CFTR dysfunction as environmental insults are limited as compared to older subjects. Studies in different age groups are required to study how well CFTR function measurements by FIS remain associated with clinical phenotypes and might lead to a better understanding of the impact of CFTR residual function and non-CFTR dependent pathways on progressive disease characteristics.

Exact FIS thresholds that distinguish between clinically relevant differences in CFTR residual function remain to be determined in follow up studies. The distribution of data of the group with more severe disease by SCC showed that the average SCC group value (106 mmol/L) was separated by 2.5 SD from the 60 mmol/L threshold, consistent with published data [13]. The corresponding group identified by FIS having more severe disease showed an average of 68,3 AUC swell units, that was separated by 11,3 SD units from the defined 1000 AUC threshold. The distribution of data from the groups identified with milder disease showed a similar distribution and relation to the used thresholds for both SCC and FIS (SCC average of 37,4 mmol/L and 1.5 SD to 60 mmol/L; FIS average of 2702 AUC and 1.7 SD to 1000 AUC). This suggests that a further refinement of CFTR residual function may be possible by FIS, which may expand the current

classification of CFTR mutations as having significant residual function or not towards a system in which no-to-minimal, low or high residual function is recognized.

For ICM, we used the validated Rotterdam-Hannover protocol and reference values (> 10 $\mu A/cm^2$) defining differences between pancreas sufficient and insufficient patients as published previously [19]. ICM protocols can differ somewhat between laboratories by type of Ussing chamber (perfused or recirculating) or pharmacological manipulations of the biopsies [17,19,20,23,24]. These studies generally found relations between ICM and clinical phenotype in CF, mostly in the context of diagnosis and pancreatic (in)sufficiency. A new European SOP for ICM is also under development, so tresholds used for ICM in this study may differ between sites and future thresholds for new SOP. As organoid technology is novel and relying on many (locally produced) media factors, additional multicenter validation is required for organoids to study site-to-site reproducibility and consistency of thresholds. The study was not designed to compare the performance of the three CFTR dependent biomarkers for prediction of individual clinical disease, but as observational study to indicate relations between FIS and clinical observations in young children with CF. In most cases, the three biomarkers of CFTR function were aligned at the individual level as to type individuals as classic or severe CF or milder forms of CF. Interestingly, FIS appeared to align with the individual clinical phenotype and the general disease liability of the genotypes in CFTR2 when ICM and SCC were not in agreement (patient 2, 4, 13, 17 and 27). It must be noted that individuals 2 and 17 only showed a marginal higher level than the ICM threshold value used here, suggesting that a slight increase in ICM threshold might have better qualified the patients in the current study. Subject 13 showed a 55 mmol/L chloride concentration that is near the 60 mmol/L SCC threshold, which was inconsistent with

ICM, FIS, genotype (F508del homozygous) and clinical phenotype. It is likely that a SCC repeat measure for subject 13 would lead to a reclassification of this patient (SCC > 60 mmol/L). Such repeat measures might also have led to a somewhat lower SCC value of subject 12 (160 mmol/L) that appears beyond human physiological limits and published data [27]. For now, it suggests that FIS appears informative for individual disease classification in the context of borderline SCC and ICM measurements or when SCC and ICM disagree, but further validation remains necessary in a larger group of patients.

ICM measurements provide fast and sensitive measurements of CFTR function in freshly isolated native tissue from CF patients that integrates the individual CFTR genotype and other genes that impact on ion transport, but also environmental factors such as CFTR modulators that can impact on CFTR function [42,43]. For patients 4 and 12, we observed relatively high ICM responses which appeared not aligned with the clinical phenotype and other biomarkers of CFTR function and largely exceed the values found in the other infants homozygous for F508del (conform the original tracings in the online supplement S6). In studies using older patient groups, such high ICM have not been reported using perfused systems [44–46], and only rarely in patient samples using recirculating Ussing chambers [47]. The unexpected high responses in ICM for the two patient samples may be age-related, or result from environmental influences on the intestinal tissue such as dietary of inflammatory components. Patient-specific mechanisms that control F508del protein maturation and apical trafficking that are not maintained in the organoid culture may also contribute [46], or the impact of cAMP/Ca^{2+}-stimulated signaling pathways on non-CFTR chloride channels [48]. As the organoid culture conditions enrich for the crypt-based secretory stem cell epithelial compartment of the

intestinal epithelium, and not the whole intestinal epithelium, the ICM-based observations may result from currents evoked by intestinal cells that are reduced in the organoid cultures such as goblet cells [49]. Clearly, additional investigations beyond the scope of the present study are needed to understand these interesting findings.

Limitations of this study include a relatively small patient cohort and limited clinical follow-up time. Moreover, a relative high number of ICM measurements were missing. Furthermore, organoid swelling is highly CFTR dependent, but not a direct readout of CFTR function, it relies on coupling of CFTR-dependent ion transport to fluid transport. Albeit that the assay is relatively straightforward and robust as compared to other in vitro readouts as indicated by 9 technical dropouts out of 1552 measurements, organoid measurements require considerable local expertise and expensive equipment. Additionally, different organoid assay conditions are used to study CFTR function in relation to wild-type CFTR [11]. The accurate typing of very low CFTR residual function also requires a different experimental setup as compared to the protocol we used here, e.g. by longer stimulations with forskolin or by measurement of luminal volume increase [50]. Most importantly, the validity of this biomarker for prediction of long term outcomes remains to be demonstrated at this point.

FIS also offers extra advantages over other biomarkers of CFTR function. Rectal biopsies can be shipped to dedicated centers for centralized and standardized analysis. FIS can also measure response to CFTR modulating therapy in a pre-treatment study, which is especially relevant for subjects with rare or unknown CFTR genotypes or questionable disease [11]. Early indicators for patients at risk for severe long term outcome might be helpful to decide when to start CFTR-

modulating therapy. Several case studies showed that *ex vivo* and *in vivo* responses to CFTR regulating therapeutics are clearly correlated [11,43,51,52], indicating that FIS assay can play a role in both timing and efficacy of drug treatment in individual patients. Furthermore, intestinal organoids can be used in the preclinical phase of CFTR modulator development [53], and after measurements of residual CFTR function and response to CFTR modulators, cells can be biobanked for future analyses without requiring further sampling and patient discomfort [54].

In conclusion, stratification for FIS of intestinal organoids identified subgroups that differed in pulmonary and gastrointestinal clinical outcome parameters. FIS correlated well with currently established CFTR-dependent biomarkers SCC and ICM. FIS appeared to be informative when SCC and ICM values were questionable and not in agreement with the clinical phenotype or CFTR genotype registry data (CFTR2). The findings in this study support that FIS in intestinal organoids is a clinically relevant biomarker of CF disease severity, and show that patient-derived stem cell resources can have value for individual disease typing in a clinical care setting.

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Table 1. Clinical characteristics of the study population at the age of 1 year (n=34)

Sex (n [%])	
Female	15 [44]
Male	19 [56]
Location of CF care (n [%])	
Utrecht	11 [32]
Rotterdam	23 [68]
Length (z-score; mean [SD])	0.15 [0.92]
Weight (z-score; mean [SD])	-0.18 [0.88]
Weight for height (z-score; mean [SD])	-0.23 [0.79]
IRT (µg/mL; median [IQR])	5.3 [2.8 – 9.9]
PAP (µg/mL; median [IQR])	149 [107.5 – 183.8]
Pancreatic sufficiency (n [%])	
Sufficient (FE > 200 µg/g)	7 [21]
Partially insufficient (FE 15-200 µg/g)	4 [12]
Insufficient (FE < 15 µg/g)	23 [67]
Pseudomonas colonization status (n [%])	
Positive	2 [6]
Negative	32 [94]
BALF (median [IQR])*	
% neutrophils	10 [4 – 22.5]
PRAGMA-CF CT score (median [IQR])‡	
% disease	3.5 [1.6 – 4.1]
Infant Lung Function test (z-score; median [IQR])†	
V'_{max}-FRC	-0.9 [-1.9 - -0.2]

SD=standard deviation; IQR=interquartile range; IRT=Immunoreactive Trypsinogen (in blood at heelprick screening); PAP=Pancreatic associated protein (in blood at heelprick screening); FE=fecal elastase; BALF=bronchoalveolar lavage fluid; PRAGMA-CF=Perth-Rotterdam Annotated Grid Morphometric Analysis for CF; V'_{max}-FRC =maximal expiratory flow at functional residual capacity. *n=19, †n=17, both only Rotterdam patients; ‡n=31

Table 2. Comparisons of clinical parameters between groups when divided in high or low values of FIS and SCC

Clinical parameter	**FIS**			**SCC**		
	Low (n=25)	**High (n=9)**	**p**	**High (n=26)**	**Low (n=8)**	**p**
Length (z-score) Mean (SD) Missing	0.15 (0.8) 0	0.14 (1.29) 0	1.00	0.07 (0.80) 0	0.39 (1.32) 0	0.54
Weight for length (z-score) Mean (SD) Missing	-0.14 (0.79) 0	-0.50 (0.79) 0	0.25	-0.19 (0.81) 0	-0.38 (0.79) 0	0.58
IRT (µg/mL) Median (IQR) Missing	**160 (137-208)** 0	**123 (86-142)** 0	**0.030**	**160 (140-207)** 0	**109 (84-138)** 0	**0.012**
PAP (µg/mL) Median (IQR) Missing	**5.9 (4.5-10.0)** 1	**3.0 (1.8-5.2)** 0	**0.039**	5.9 (3.7-9.9) 1	3.9 (2.0-4.8) 0	0.053
Pancreatic sufficiency (n,%) - Sufficient (FE > 200 µg/g) - Part. insufficient (FE 15-200 µg/g) - Insufficient (FE <15 µg/g) Missing	**2 (8)** **4 (16)** **19 (76)** 0	**7 (78)** **0 (0)** **2 (22)** 0	**<0.001**	**2 (8)** **4 (15)** **20 (77)** 0	**7 (88)** **0 (0)** **1 (12)** 0	**<0.001**
Leeds score (accumulated) Median (IQR) Missing	6.0 (4.0-12.0) 0	4.0 (3.0-8.0) 0	0.26	6.0 (4.0-12.0) 0	3.5 (2.0-5.0) 0	0.051
BALF % neutrophils Median (IQR) Missing	10.0 (4.0-23.8) 11	10.0 (9.0-13.0) 4	0.82	10.0 (4.0-20.5) 12	10.0 (9.0-22.0) 3	0.96
PRAGMA-CF score % disease Median (IQR) Missing	**3.6 (2.4-4.9)** 1	**1.8 (0.8-2.3)** 2	**0.049**	**3.7 (2.4-4.8)** 1	**1.3 (0.6-2.0)** 2	**0.007**
V'max-FRC (z-score) Median (IQR) Missing	**-1.9 (-2.5--0.9)** 15	**-0.2 (-1.0--0.1)** 2	**0.033**	-1.0 (-2.4--0.5) 15	-1.0 (-1.6--0.3) 2	0.46

Table 2. Comparison of values of clinical parameters when the group is divided in low vs high FIS values or high vs low SCC values, respectively. See text and figure 3 for borders between high and low values for FIS and SCC. FIS=Forskolin induced swelling; SCC=Sweat chloride concentration; IRT=Immunoreactive trypsinogen; PAP=Pancreatic associated protein; FE=fecal elastase; Leeds score: accumulated colonization status of different CF pathogens (Pseudomonas *aeruginosa*, Staphylococcus *aureus*, Haemophilus *influenzae*); BALF=bronchoalveolar lavage; PRAGMA-CF=Perth-Rotterdam Annotated Grid Morphometric Analysis for CF; V'_{max}-FRC =maximal expiratory flow at functional residual capacity.

Figure legends

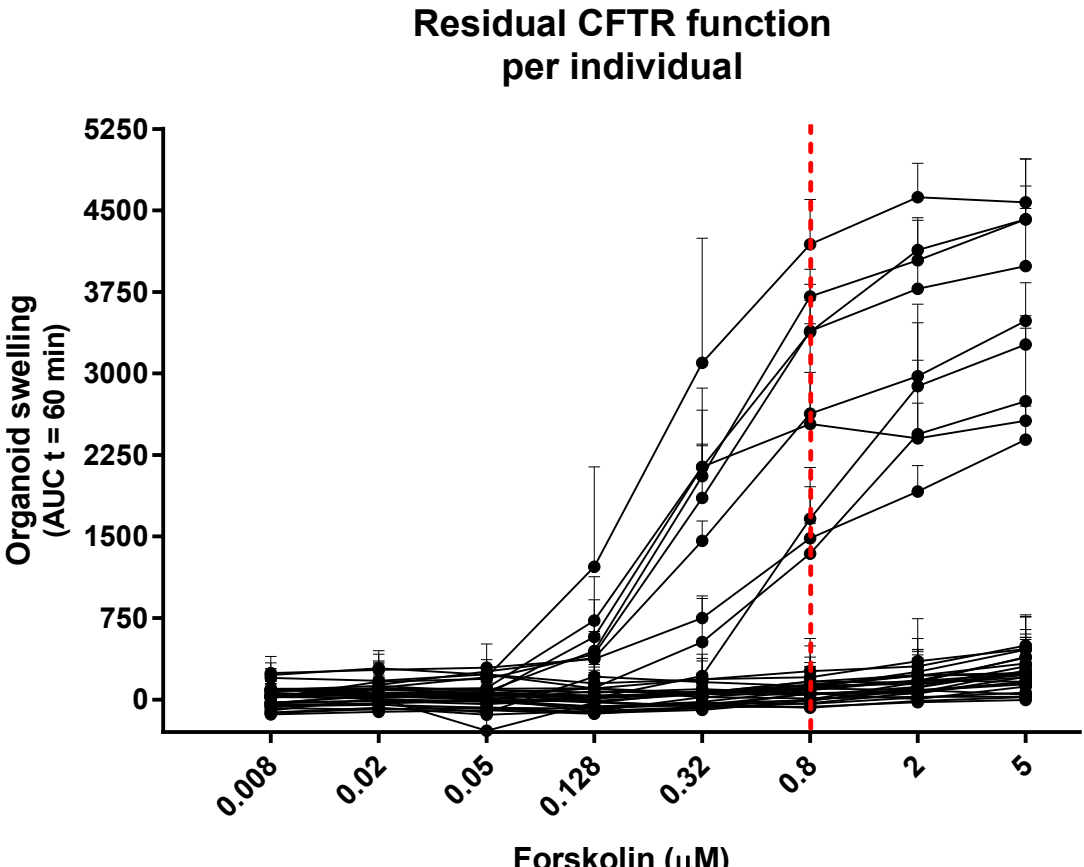
Figure 1a. Forskolin induced swelling of organoids of all patients with various mutations expressed as the absolute area under the curve (AUC) at t=60 min, mean ± SD. The red dotted line indicate FIS values at 0.8µM forskolin, see text.

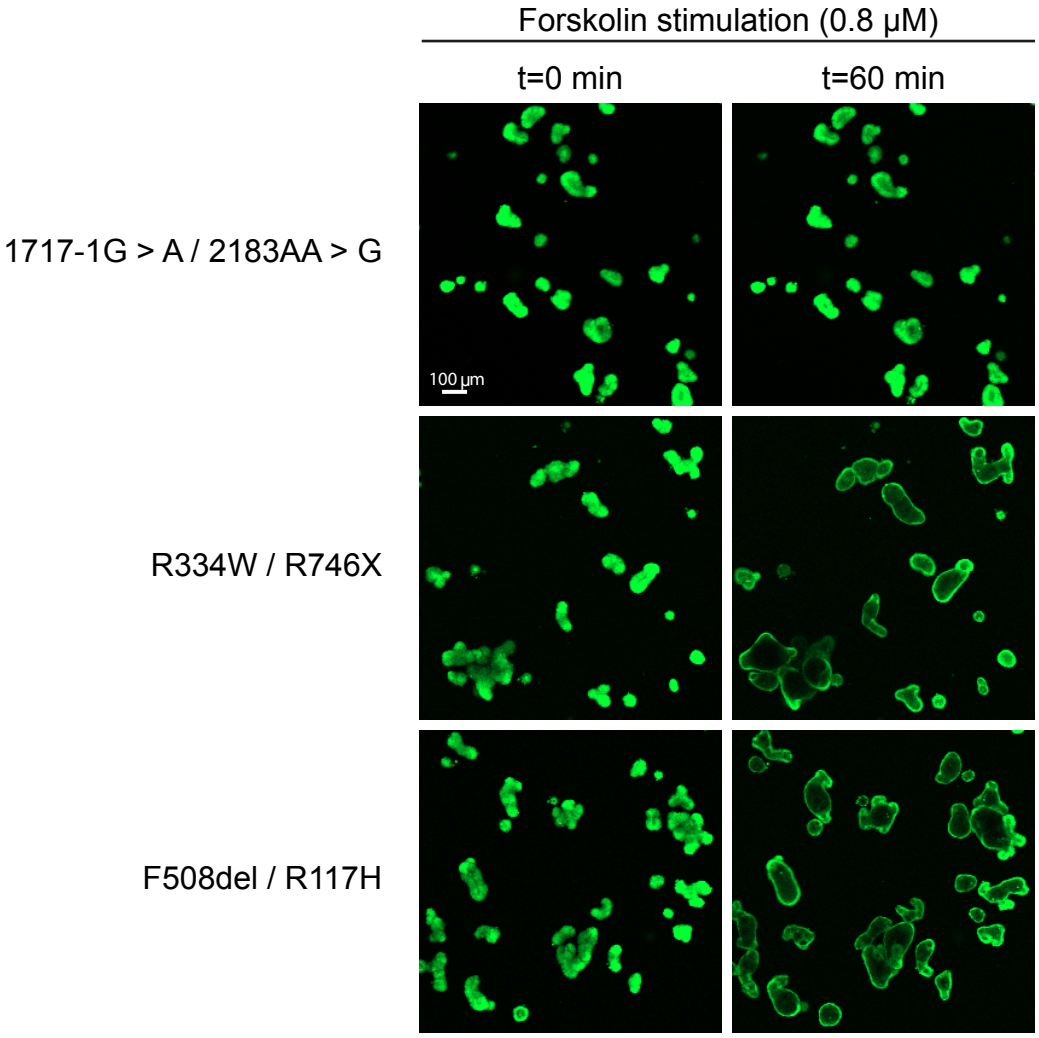
Figure 1b. Representative confocal microscopy images of calcein-green labeled organoids of 3 CF subjects with different mutations, before and 60 min. after stimulation with 0.8µM forskolin. Scale bar = 100 µm.

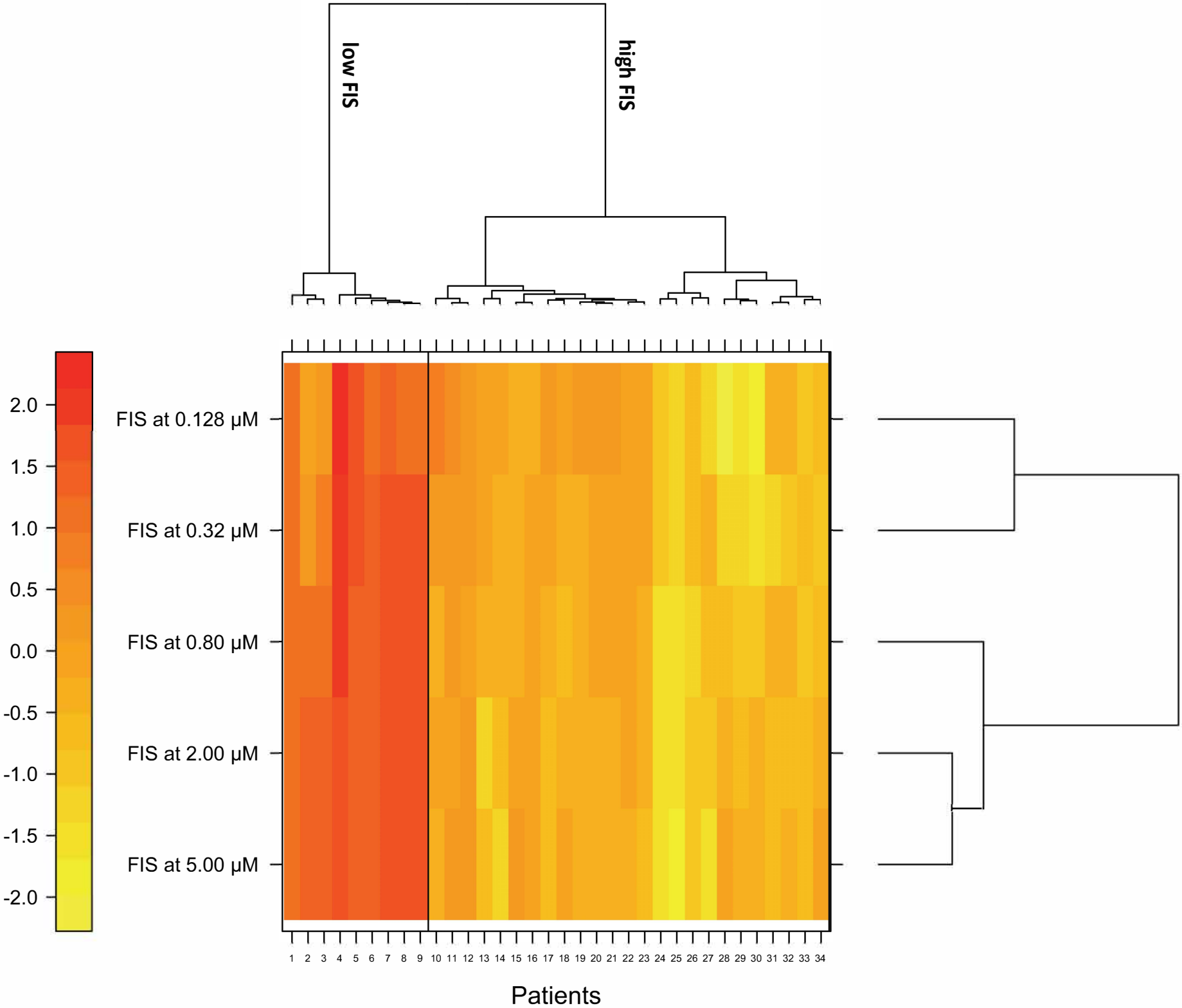
Figure 1c. Cluster analysis of FIS responses of all different forskolin concentrations of all patients. Forskolin concentrations of 0.128 – 5.0 µM are used as variables. Two different groups are identified, FIS high (n= 9) and FIS low (n= 25).

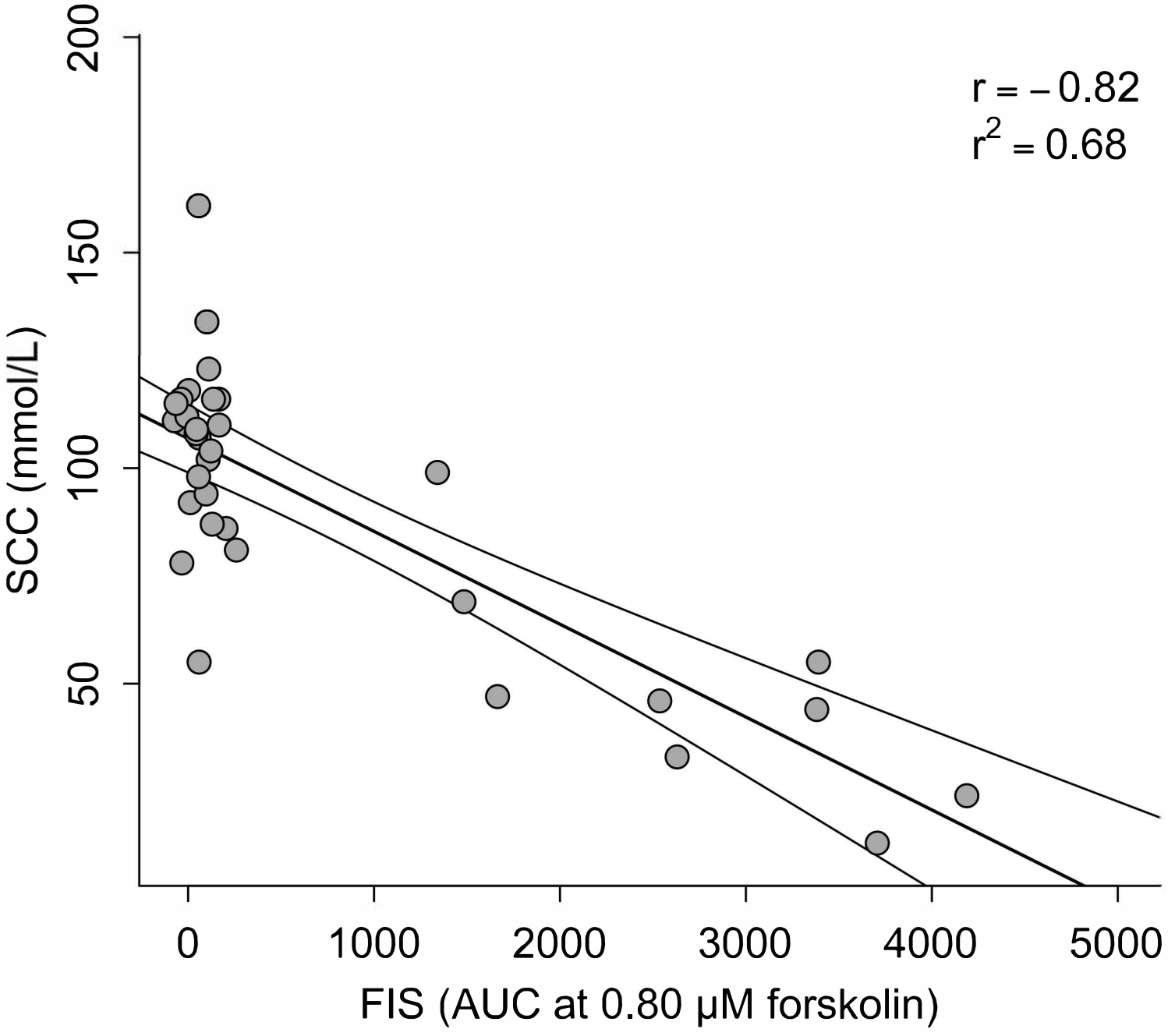
Figure 2. Pearson correlations of sweat chloride concentration (SCC; Fig. 2a, n=34) or intestinal current measurements (ICM, ΔI_{sc} (carbachol+cAMP/forsk+histamine); Fig. 2b, n=23) versus forskolin induced swelling (FIS) at 0.8µM forskolin. Each dot represents one individual.

Figure 3 a,b,c. Results of different CFTR bioassay measurements per individual. (a) Forskolin-induced swelling of organoids at 0.8 µM forskolin. (b) Sweat Chloride Concentrations. (c) Intestinal current measurements, ΔI_{sc} (carbachol+cAMP/forsk+histamine) n=23. Dotted lines indicate borders between high and low values (a) or positive and intermediate levels (b,c), see results section. ND=not determined.


Residual CFTR function
per individual
Organoid swelling
(AUC t = 60 min)
5250
4500
3750
3000
2250
1500
750
0
0.008
0.02
0.05
0.128
0.32
0.8
2
5
Forskolin (μM)


Forskolin stimulation (0.8 μM)
t=0 min
t=60 min
1717-1G > A / 2183AA > G
100 μm
R334W / R746X
F508del / R117H


low FIS
high FIS
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
FIS at 0.128 µM
FIS at 0.32 µM
FIS at 0.80 µM
FIS at 2.00 µM
FIS at 5.00 µM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Patients


r = −0.82
r² = 0.68
SCC (mmol/L)
200
150
100
50
0
1000
2000
3000
4000
5000
FIS (AUC at 0.80 μM forskolin)

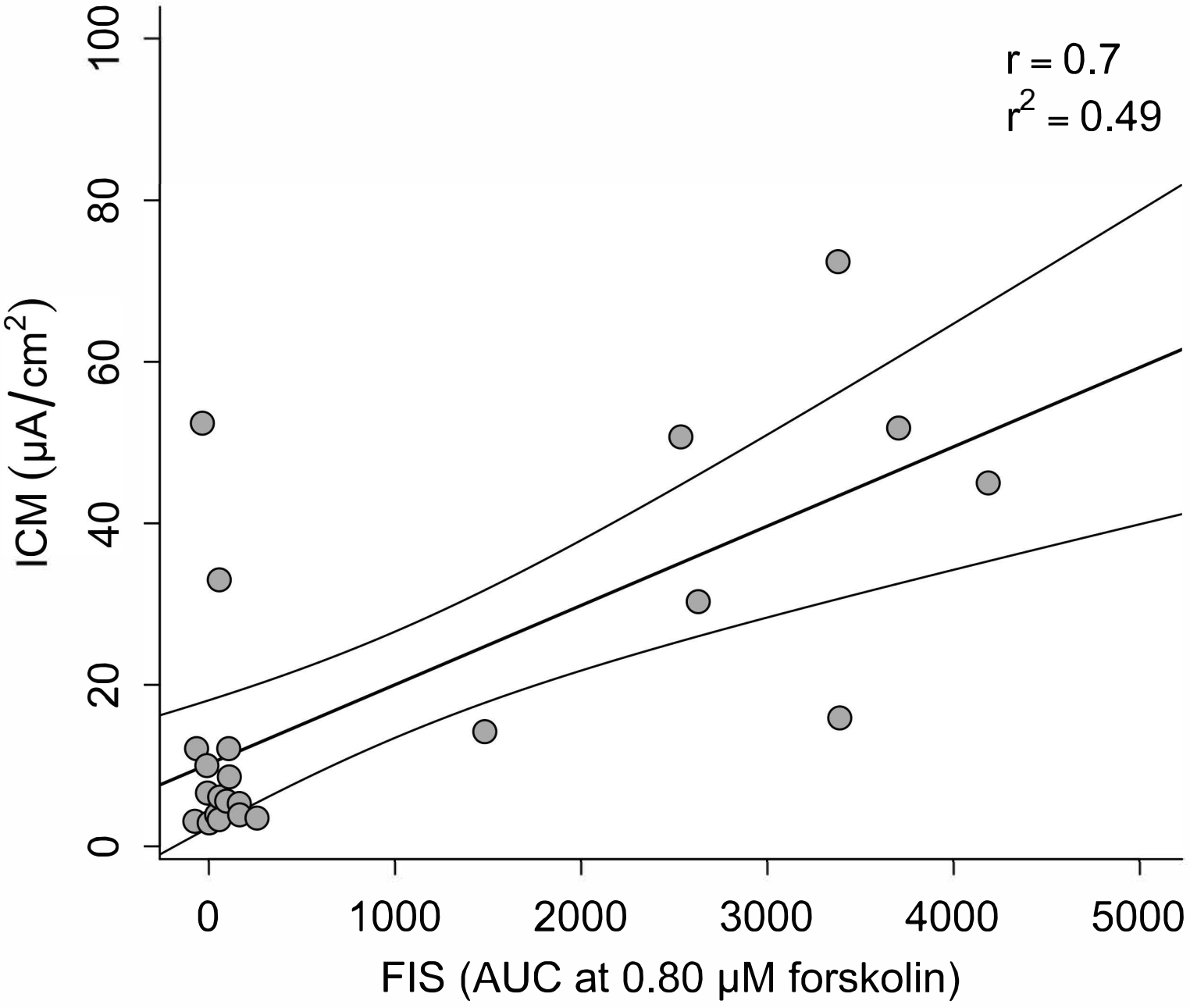

r = 0.7
r2 = 0.49
ICM (μA/cm2)
0
20
40
60
80
100
0
1000
2000
3000
4000
5000
FIS (AUC at 0.80 μM forskolin)

fig 3a. Forskolin induced swelling per individual

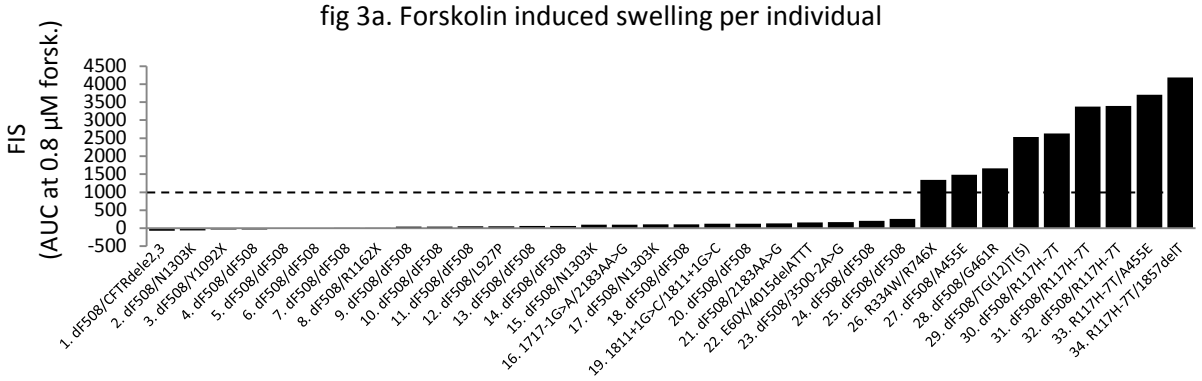


fig 3b. Sweat chloride concentration per individual

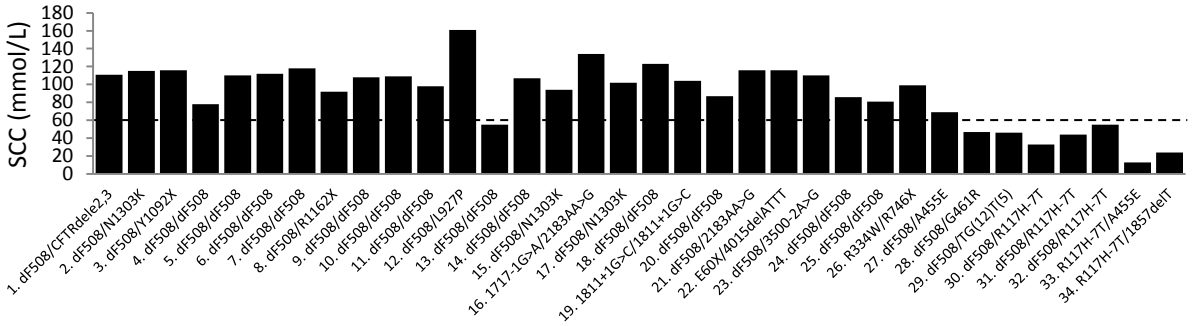
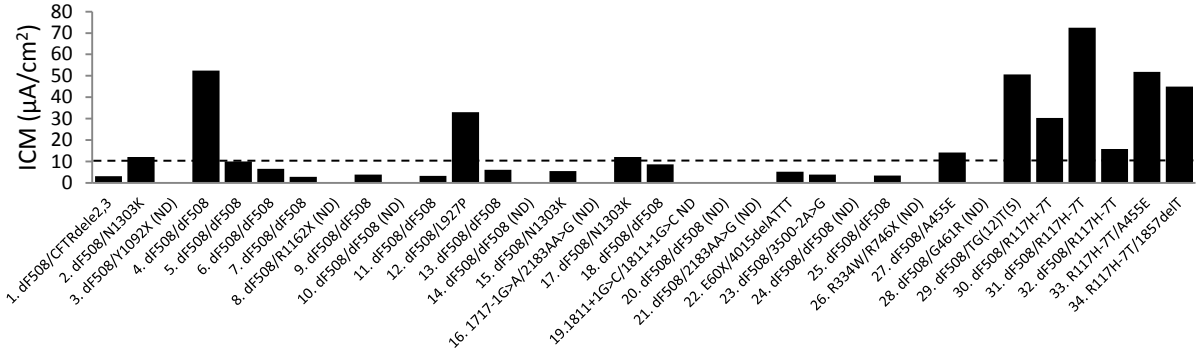


fig 3c. Intestinal current measurement per individual



S1. Monitoring protocol after positive newborn screening for CF

(months of age)	0	1	2	3	4	5	6	7	8	9	10	11	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
1. Informed consent	x																												
2. Demographic History	x																												
3. DNA	x																												
4. Sweattest	x																												
5. Out-patient clinic visits/contacts	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
a. Pediatric pulmonogist/CF nurse	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
b. Gastro-enterologist/dietician		x	x°	x			x				x°		x°				x°				x°				x°				x°
6. Medical history/review symptoms	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
7. Physical examination	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
8. Length, weight, head circumference	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
9. Symptom diary	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
10. Throat swab/sputum culture, nasopharyngeal swab	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
11. Faecal elastase	x												X#				X#				X#				X#				X#
12. Faecal fat balance																									x				x
13. Blood				x									x				x				x				x				x
14. Urine				x*									x*																
15. Bronchoscopy and BAL													x								x								x
16. Chest X-ray				x													x								x				
17. Chest CT scan													x								x								x
18. Infant lungfunction¥													x																
19. Spirometry																									x	x	x	x	x
20. CFQ-R																													x
21. ICM													x																x^

* for Na, creatinine
# only in pancreatic sufficient patients
° both GI specialist and dietician, other months only dietician
^ in case of rest activity measured at 12 months
¥ only applicable for Rotterdam patients

S2. CT procedures

All CTs of CF subjects were made supine, with pressure control under general anesthesia (for Utrecht patients) or with free-breathing (for Rotterdam patients). To reduce atelectasis in sedated patients, alveolar recruitment maneuvers were performed consisting of 10 consecutive slow breath with inspirations pressure of 40 cm H_2O followed by a breath hold with end-inspiration pressure of 25 cm H_2O. Volumetric end-inspiratory acquisitions were obtained using a Philips Brilliance 16p scanner in Utrecht and Siemens Definition Flash scanner in Rotterdam.

See also Kuo et al. Ped Pulmonol 2017

CT setting	**Rotterdam**	**Utrecht**
CT Scanner	Siemens Definition Flash	Philips Brilliance 16p
Scan type	Inspiratory	Inspiratory
Acquisition	Volumetric	Helical
Rotation time (ms)	280	500
Pitch	3	0.9
Slice thickness (mm)	1.0	1.0
Increment (mm)	0.7	0.7
Collimation (mm)	0.63	16x0.75
Tube voltage (kVp)	100	90
Current-time product (mAs)	10	20
CTDIvol (mGy)	0.5	0.7
Dose-Length Product (mGy cm)	12.3	12.1

Table S2. CT settings. CTDIvol and Dose-Length Product based on a 32-cm phantom and reported as mean.

S3. Intestinal current measurements (ICM)

Rectal biopsies (4 per individual; ~12 months of age) were collected with a suction biopsy device (Model Meekers Medical 790166, Utrecht, Netherlands). The electrogenic transport of ions across the intestinal epithelium was measured as short circuit current (Isc) by a standardized protocol used by the CF centers in Rotterdam/Utrecht and Hannover [Derichs et al. Thorax 2010 and De Jonge et al. JCF 2004]. In brief, the biopsies were mounted in tissue sliders (aperture 1.13 mm2), inserted in recirculating Ussing chambers, and incubated at 37°C with Meyler buffer solution gassed with 95% O2, 5% CO2. After equilibration for 20 min, the basal potential difference, transepithelial resistance and short-circuit current (Isc) were determined by a voltage clamp-amplifier (DVC-1000, WPI) and a PowerLab digitalizer (AD Instruments). Next, compounds were added in a standardized order to either the mucosal (M) or the serosal (S) side of the epithelial tissue and Isc response to these additions was registered: amiloride (100 µM, M); carbachol (100 µM, S); DIDS (200 µM, M); histamine (500 µM, S), and 8-bromo-cAMP (1 mM, M+S)/forskolin (10µM, S). Finally, the transepithelial resistance and potential difference were measured to verify tissue viability. Crude Isc values (µA) were converted to $\mu A/cm^2$ on the basis of the surface area of the aperture, and the maximal individual ΔIsc responses after stimulation with specific substances were averaged from all biopsies without technical problems (3-4 per subject). The cumulative value of the average current increase provoked by the secretagogues carbachol, cAMP/forskolin, and histamine (ΔIsc, carb+cAMP+hista), mainly reflecting electrogenic, CFTR mediated chloride secretion, rather than the response to cAMP/forskolin alone (as monitored in the FIS assay), was used for

analyses in this study. As described in the paper of Derichs et al. (19) we used a cut-off value for subjects with CF-PI of <10 $\mu A/cm^2$.

S4. Organoid culture from rectal biopsies and forskolin-induced swelling assay (FIS)

Crypts were isolated from rectal biopsies and seeded in 50% Matrigel (growth factor-reduced and phenol-free; Corning) in 24-well plates (~10 to 30 crypts in three 10-µl Matrigel droplets per well). Growth medium [Sato et al, Gastroenterology 2011; Dekkers et al, Nature Med 2013] was further supplemented with Primocin (1:500; Invivogen). Vancomycin and gentamycin (Sigma) were added during the first week of culture to prevent infection. The medium was refreshed every 2 to 3 days, and organoids were passaged ~1:5 every 7 to 10 days. In short, intestinal organoids (passages 1 to 20) from a 7- to 10-day old culture were seeded in 96-well culture plates (Greiner) in 50% Matrigel containing 20 to 80 organoid structures and immersed overnight in 50 µl organoid culture medium. One day after seeding, organoids were incubated for 30 minutes with 3 µM calcein green (Invitrogen) and stimulated with forskolin (Sigma). To assess individual level of residual CFTR function in intestinal organoids, organoid swelling was induced by stimulating organoids with various forskolin concentrations. The 96-well plates were directly analyzed by confocal live cell microscopy (LSM 800, Zeiss) (all experiments were performed at 37°C, 5% CO_2). Per well, the total organoid area (xy plane in μm^2) increase relative to t = 0 of forskolin treatment was quantified using Zen Image analysis software module (Zeiss). Area under the curve (AUC) (t = 60 min; baseline, 100%) was calculated using Excel.

All forskolin concentrations were measured in duplicate. 21 out of 34 organoid cultures were measured at three independent culture time points, 9 cultures were measured at two culture timepoints and 4 cultures were measured at four independent time points. Sporadically, we

measured only 6 instead of 8 forskolin concentrations due to cell availability. For the 34 organoid lines, we generated 1552 data points and censored 9 data points as they were qualified as extreme outliers (>6SD as compared to the average of all replicates) due to technical reasons related to imaging.

S5. Overview of all chloride secretory responses as measured with ICM (in biopsies of 23 of 34 patients)

Patient number	$\Delta I_{sc,carbachol}$ ($\mu A/cm^2$)	$\Delta I_{sc,cAMP/forskolin}$ ($\mu A/cm^2$)	$\Delta I_{sc,histaminel}$ ($\mu A/cm^2$)	$\Delta I_{sc,carb+cAMP/fors+hist}$ ($\mu A/cm^2$)
1	0±0.6	0±0.7	3.1±0.3	3.1
2	0±4.5	3.1±3	0±0.5	3.1
3	-	-	-	-
4	22.1±10.4	11.3±5	19±5.6	52.4
5	8.2±2.5	1.1±1.6	0.7±10.1	10
6	0±2.4	3.3±0.7	3.3±0.3	6.6
7	0±4.4	2.9±0.9	0±8.5	2.9
8	-	-	-	-
9	0±4.0	1.7±2.1	2.2±1.0	3.9
10	-	-	-	-
11	0±7.3	3.3±4.0	0±2.2	3.3
12	11.5±4.5	11.3±11.1	10.2±6.3	33
13	0±2.0	6.1±2.5	0±2.8	6.1
14	-	-	-	-
15	0±9.0	2.9±2.9	2.7±1.2	5.6
16	-	-	-	-
17	0±3.8	3.4±1.1	8.7±3.5	12.1
18	0±1.1	1.8±1.4	6.8±2.2	8.6
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	5.3±8.6	0±6.7	0±2.2	5.3
23	0±9.6	3.9±0.8	0±5.5	3.9
24	-	-	-	-
25	0±1.3	3.3±1.3	0.2±3.0	3.5
26	-	-	-	-
27	0±5.6	14.2±3.8	0±5.8	14.2
28	-	-	-	-
29	13.2±10.8	21.8±10.3	15.7±18.7	50.7
30	2.3±1.5	23±4.3	5±0.4	30.3
31	34.8±14.8	32.2±3.6	5.4±3.3	72.4
32	0±7.6	9.5±4.4	6.4±3.8	15.9
33	23±23.5	17.3±13.5	11.5±6.1	51.8
34	15.1±15.8	29.9±15.5	0±7.4	45

ICM: intestinal current measurement. Isc: short-circuit current. Negative currents (reflecting apical K^+ secretion) are set to 0. Mean ± SD are given. Hyphens indicate that ICM values are not determined.