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

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

We investigated whether FIS corresponds with clinical outcome parameters and biomarkers of CFTR function in 34 infants diagnosed with CF. Relationships with FIS were studied for indicators of pulmonary and gastrointestinal disease.

Children with low FIS had higher levels of immunoreactive trypsinogen (p=0.030) and pancreatitis-associated protein (p=0.039), more often had pancreatic insufficiency (p<0.001), had more abnormalities on chest computed tomography (p=0.049), and had 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 r=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.

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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 (leucine-rich-repeat-containing G-protein-coupled receptor 5) as a 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, multi-lineage 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 characterised by accumulation of viscous mucus in the pulmonary and gastrointestinal tracts, resulting in bacterial infections, chronic inflammation and malnutrition. A high degree of variability in organ dysfunction and survival exists between subjects with CF, which can be caused by variation in the *CFTR* gene itself, as over 2000 *CFTR* variants have been reported in the Cystic Fibrosis Mutation Database (www .genet.sickkids.on.ca/cftr/app), as well as by additional genetic and environmental factors [12]. Hence, clinicians face great difficulties when predicting the clinical course of the individual patient based on the CFTR genotype, especially for subjects carrying rare CFTR variants [13]. The Clinical and Functional TRanslation of CFTR (CFTR2) project (www.CFTR2.org) aims to provide functional and clinical information on CFTR mutations.

Individual biomarkers of CFTR function play an important role for diagnosing CF or other CFTR-related diseases [14]. *In vivo* sweat chloride concentration (SCC) measurements 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, leading to thresholds for CF diagnosis and disease severity classification. These studies have 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 relationships 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 (both in the Netherlands) enrolled in a standardised monitoring protocol, adapted from the protocol of the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST-CF) [29, 30]. The Dutch protocol for newborn screening for CF includes measurement of immunoreactive trypsinogen (IRT) and pancreatitis-associated protein (PAP) in heel prick blood [31]. The monitoring protocol includes a SCC test and CFTR genotyping after birth, and bacterial airway cultures at every regular outpatient visit. At 1 year of age, patients underwent general anaesthesia for chest computed tomography (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). For a detailed description of the monitoring protocol, see supplementary material 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 the European Cystic Fibrosis Society Clinical Trials Network (ECFS-CTN), and both Utrecht and Rotterdam are certified centres to perform sweat tests [18, 32]. ILFT, chest CT, bronchoscopy and BAL were performed according to standardised procedures [33–35]. For ILFT, measurements of forced expiration using rapid thoraco-abdominal compression techniques and the forced deflation technique were used. For CT procedures, see supplementary material S2. Severity of airway disease (% disease) was scored in random order, blinded to patient identifiers using the Perth–Rotterdam Annotated Grid Morphometric Analysis for CF (PRAGMA-CF) CT scoring method. The % disease reflects the percentage of total lung volume showing bronchiectasis, bronchial thickening or mucus plugging [36]. Bronchoscopy was performed under general anaesthesia. BAL was done with three aliquots of 1 $mL \cdot kg^{-1}$ of NaCl 0.9% each in the right middle lobe and one aliquot in the most affected lobe. ICMs in rectal biopsies were performed using a standardised protocol [19, 37] (for a detailed description, see supplementary material S3). The cumulative response to carbachol, forskolin and histamine was used for analyses.

Methods for generation of intestinal organoids and measurement of FIS were slightly adapted from protocols described previously [9, 11, 38]. Additional detail on these methods is provided in supplementary material 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, starting 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 (R Project; www.r-project.org) was used for all analyses and p-values were reported based on two-sided tests.

Results

Between May 2011 and 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

TABLE 1 Clinical characteristics of the study population at the age of 1 year

Patients	34
Sex	
Female	15 (44)
Male	19 (56)
Location of CF care	
Utrecht	11 (32)
Rotterdam	23 (68)
Length z-score	0.15±0.92
Weight z-score	−0.18±0.88
Weight for height z-score	−0.23±0.79
IRT μg·mL^{-1}	5.3 (2.8–9.9)
PAP μg·mL^{-1}	149 (107.5–183.8)
Pancreatic sufficiency	
Sufficient (FE >200 μg·g^{-1})	7 (21)
Partially insufficient (FE 15–200 μg·g^{-1})	4 (12)
Insufficient (FE <15 μg·g^{-1})	23 (67)
***Pseudomonas* colonisation status**	
Positive	2 (6)
Negative	32 (94)
BALF % neutrophils[#]	10 (4–22.5)
PRAGMA-CF CT score % disease[¶]	3.5 (1.6–4.1)
Infant lung function test V'_{max}-FRC z-score[+]	−0.9 (−1.9– −0.2)

Data are presented as n, n (%), mean±SD or median (interquartile range). CF: cystic fibrosis; IRT: immunoreactive trypsinogen (in blood at heel prick screening); PAP: pancreatitis-associated protein (in blood at heel prick screening); FE: faecal elastase; BALF: bronchoalveolar lavage fluid; PRAGMA-CF: Perth–Rotterdam Annotated Grid Morphometric Analysis for CF; CT: computed tomography; V'_{max}-FRC: maximal expiratory flow at functional residual capacity. [#]: n=19, only Rotterdam patients; [¶]: n=31; [+]: n=17, only Rotterdam patients.

(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 supplementary material S5.

Forskolin dose-dependently induced swelling of organoids in a patient- and CFTR-genotype-dependent fashion (figures 1 and 2). In total, we measured 1552 data points and censored nine points as these were qualified as extreme outliers (>6 SD difference from the average of the experimental replicates). Only forskolin concentrations ⩾0.128 μM 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 (high FIS n=9, low FIS n=25; figure 3). These two groups could also be accurately identified in figure 1 at forskolin concentrations ⩾0.8 μM with an area under the curve (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 *versus* 123 μg·mL^{-1}; p=0.030) and PAP concentrations (5.9 *versus* 3.0 μg·mL^{-1}; p=0.039), more frequently had pancreatic insufficiency (19 out of 25 *versus* two out of nine patients; p<0.001), had higher PRAGMA-CF CT scores for % disease (3.6 *versus* 1.8; p=0.049) and lower z-scores for maximal expiratory flow at functional residual capacity (−1.9 *versus* −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=1.97×10^{-9}; figure 4a) and with *ex vivo* ICM (r=0.70 (95% CI 0.41–0.86; p=1.93×10^{-4}; figure 4b). We conclude from this that 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 significantly lower number of observations may introduce a biased interpretation. To divide groups with high and low SCC values into severe or milder phenotypes of CF, we used the generally accepted SCC borderline of 60 mmol·L^{-1} [18, 40, 41]. Patients with high SCC values (>60 mmol·L^{-1}) compared to patients with low SCC values had higher IRT concentrations (160 *versus* 109 μg·mL^{-1}; p=0.012), more frequently had pancreatic insufficiency (20 out of 26 *versus* one out of eight patients; p<0.001) and had higher PRAGMA-CF CT scores for % disease (3.7 *versus* 1.3; p=0.007). The subgroups identified by high or low FIS or SCC values differed by three

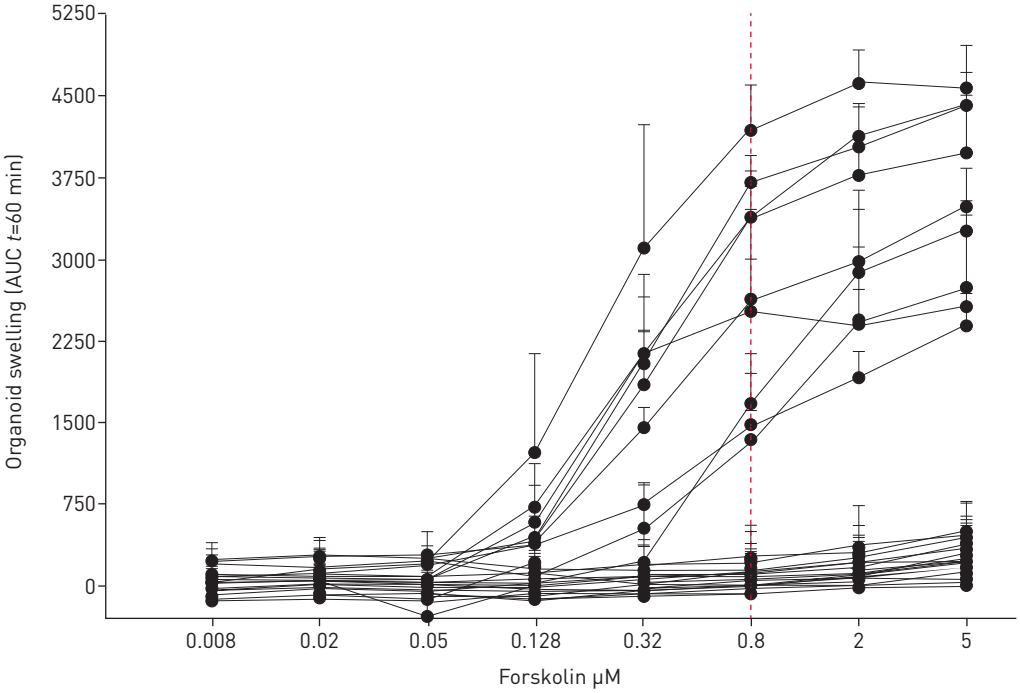

FIGURE 1 Forskolin-induced swelling (FIS) of organoids of all patients with various mutations, expressed as the absolute area under the curve (AUC) at *t*=60 min, presented as mean±SD. The red dotted line indicates FIS values at 0.8 µM forskolin (see main text).

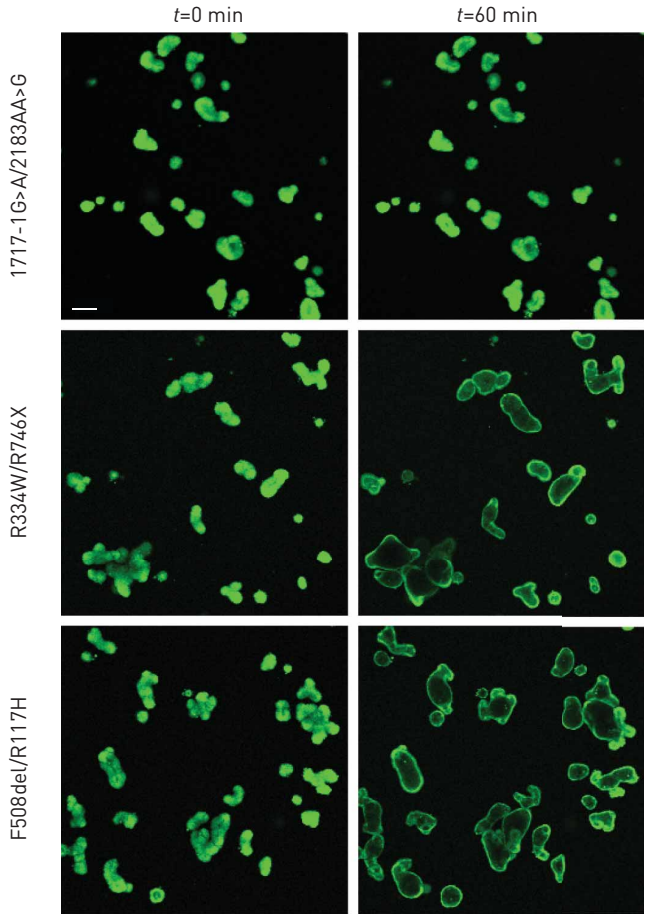

FIGURE 2 Representative confocal microscopy images of calcein green labelled organoids of three cystic fibrosis subjects with different mutations, before and 60 min after stimulation with 0.8 µM forskolin. Scale bar=100 µm.

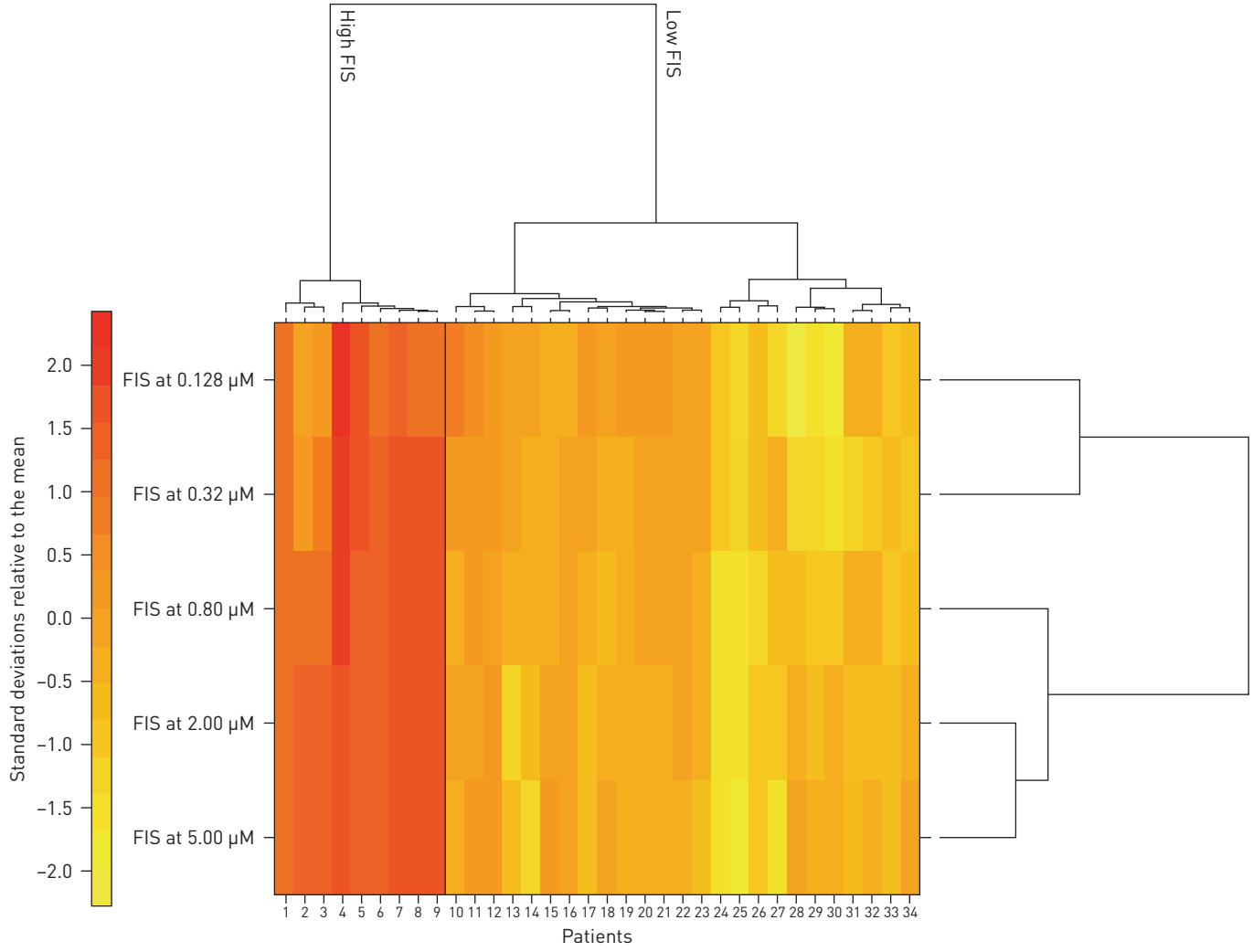

FIGURE 3 Cluster analysis of forskolin-induced swelling (FIS) responses of all different forskolin concentrations of all patients. Forskolin concentrations of 0.128–5.0 µM were used as variables. Two different groups were identified, high FIS (n=9) and low FIS (n=25).

individuals (numbers 13, 26 and 27) in this cohort. In conclusion, FIS-based subgroups associated with five out of nine studied clinical end-points during the first year of life, in comparison to three for SCC-based subgroups.

These 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 to discriminate between low or high residual CFTR function (for FIS) or mild or severe CF phenotype (for SCC and ICM, using 60 mmol·L^{-1} and 10 µA·cm^{-2} as the thresholds, respectively) [19]. FIS shows a clear dichotomous distribution of data (figure 5a), in contrast to more gradually dispersed values of SCC and ICM (figure 5b and c). In general, low FIS values corresponded with SCC >60 mmol·L^{-1} and ICM values <10 µA·cm^{-2}, but some discrepancies were observed. For example, individual number 4, with a clinically 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 (patient number 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 (according to CFTR2), pancreatic insufficiency (faecal elastase <15 µg·g^{-1}) and need for eight antibiotic treatments because of pulmonary symptoms in the first year of life. Two other individuals with SCC >60 mmol·L^{-1} (numbers 26 and 27) showed high FIS levels (the ICM was also high for number 27 and not available for number 26). These subjects also displayed a milder phenotype as illustrated by pancreatic sufficiency (faecal elastase >500 µg·g^{-1}) 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.

TABLE 2 Comparisons of clinical parameters between groups when divided into high or low values[#] of forskolin-induced swelling (FIS) and sweat chloride concentration (SCC)

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

IRT: immunoreactive trypsinogen; IQR: interquartile range; PAP: pancreatitis-associated protein; FE: faecal elastase; BALF: bronchoalveolar lavage fluid; PRAGMA-CF: Perth–Rotterdam Annotated Grid Morphometric Analysis for CF; CT: computed tomography; *V*′$_{max}$-FRC: maximal expiratory flow at functional residual capacity. [#]: see text and figure 3 for borders between high and low values for FIS and SCC; [¶]: accumulated colonisation status of different cystic fibrosis pathogens (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*). Bold indicates significance at p<0.05.

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 CF. 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 such 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 >0.128 μM convincingly divided this consecutive group of infant CF patients into two separate groups. FIS at 0.8 μM forskolin precisely identifies these two clusters, and it therefore seems sufficient to measure FIS values at fewer forskolin concentrations for typing CFTR residual function. Impressively, the cluster analysis of FIS data of only a small number 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].

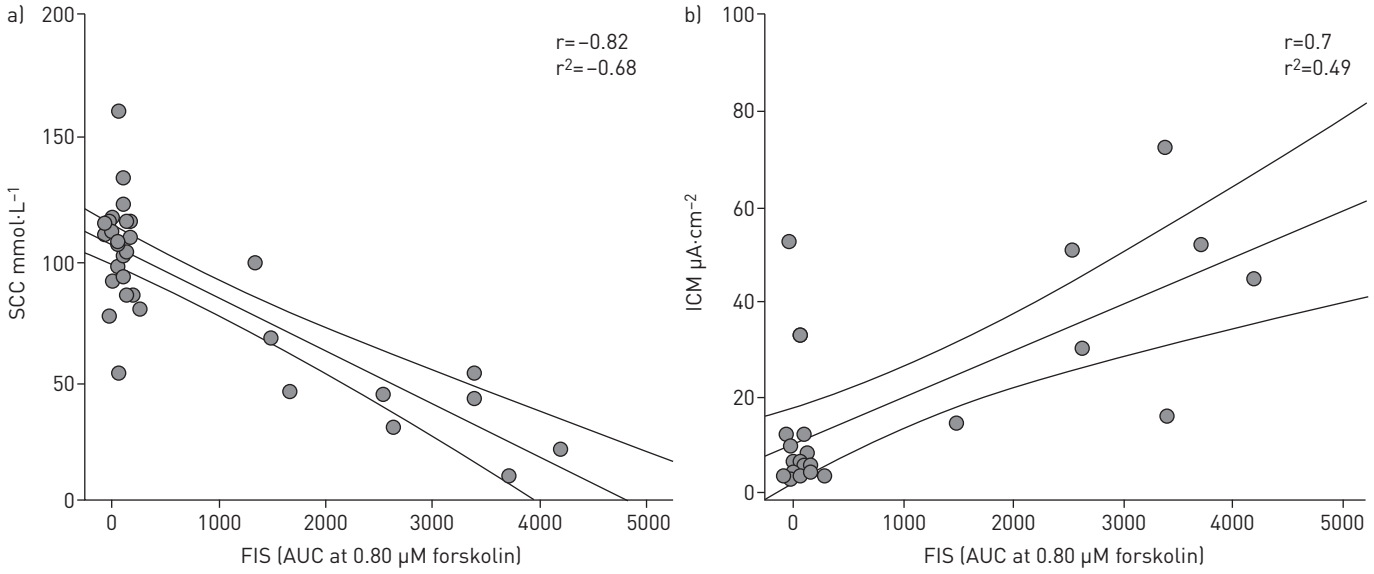

FIGURE 4 Pearson correlations of a) sweat chloride concentration (SCC; n=34) and b) intestinal current measurement (ICM; cumulative change in short-circuit current due to carbachol, cAMP/forskolin and histamine; n=23) *versus* forskolin-induced swelling (FIS) at 0.8 µM forskolin. Each dot represents one individual. AUC: area under the curve.

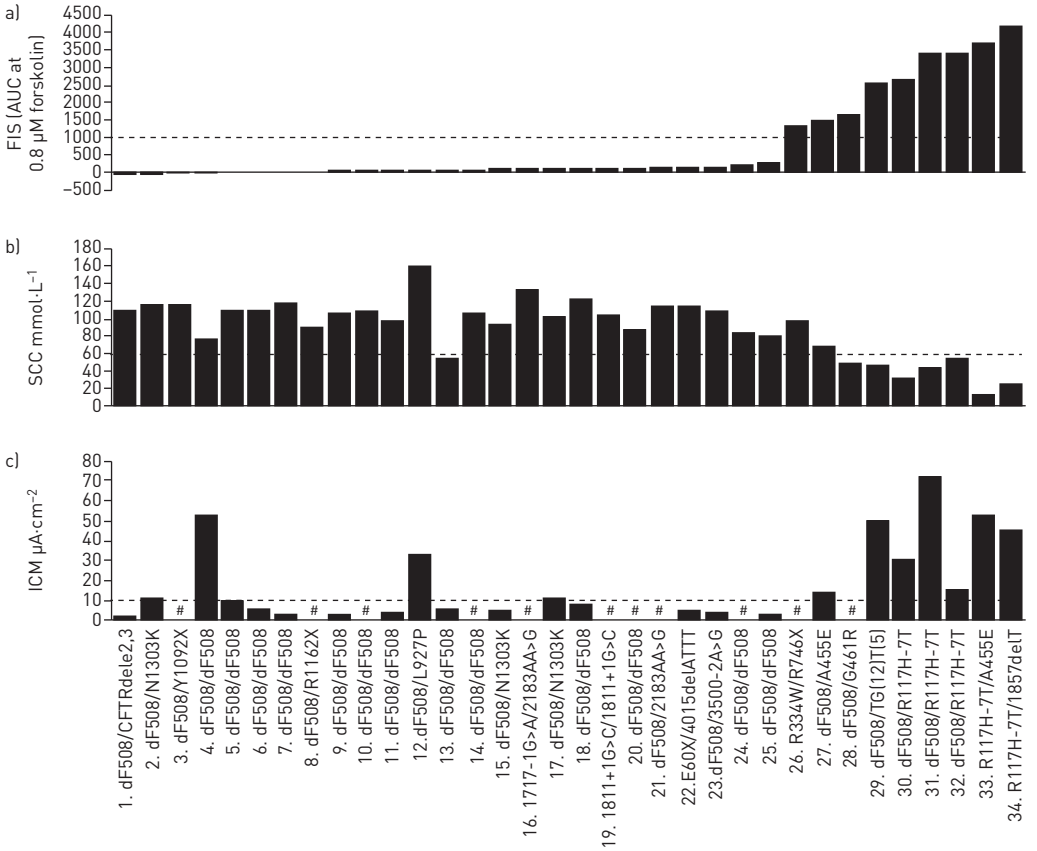

FIGURE 5 Results of different cystic fibrosis transmembrane conductance regulator (CFTR) bioassay measurements for each individual. a) Forskolin-induced swelling (FIS) of organoids at 0.8 µM forskolin. AUC: area under the curve. b) Sweat chloride concentration (SCC). c) Intestinal current measurement (ICM), the cumulative change in short-circuit current due to carbachol, cAMP/forskolin and histamine (n=23). Dotted lines indicate borders between a) high and low values or b, c) positive and intermediate levels (see results section). #: not determined.

The most important finding in this study is that CFTR function as measured by FIS has been demonstrated 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 changes 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 in comparison 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 mean SCC group value (106 mmol·L^{-1}) was separated by 2.5 SD from the 60 mmol·L^{-1} threshold, consistent with published data [13]. The corresponding group identified by FIS as having more severe disease showed a mean of 68.3 AUC swell units, which 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 mean of 37.4 mmol·L^{-1} and 1.5 SD to 60 mmol·L^{-1}; FIS mean 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 recognised.

For ICM, we used the validated Rotterdam–Hannover protocol and reference values (>10 μA·cm^{-2}), defining differences between patients with pancreatic sufficiency and insufficiency, 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 have generally found relationships between ICM and clinical phenotype in CF, mostly in the context of diagnosis and pancreatic (in)sufficiency. A new European standard operating procedure for ICM is also under development, so thresholds used for ICM in this study may differ between sites and differ from future thresholds for the new standard operating procedure. As organoid technology is novel and relies on many (locally produced) media factors, additional multicentre 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 an observational study to indicate relationships 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 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 (patients 2, 4, 13, 17 and 27). It must be noted that individuals 2 and 17 only showed a marginally 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^{-1} chloride concentration that is near the 60 mmol·L^{-1} 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^{-1}). Such repeat measures might also have led to a somewhat lower SCC value for subject 12 (160 mmol·L^{-1}), which appears beyond human physiological limits and published data [27]. For now, our data suggest that FIS appears informative for individual disease classification in the context of borderline SCC and ICM or when SCC and ICM disagree, but further validation remains necessary in a larger group of patients.

ICM provides fast and sensitive measurement of CFTR function in freshly isolated native tissue from CF patients, which integrates the individual CFTR genotype and other genes that have an impact on ion transport, and also environmental factors such as CFTR modulators that can affect CFTR function [42, 43]. For patients 4 and 12, we observed relatively high ICM responses that appeared not to be aligned with the clinical phenotype and other biomarkers of CFTR function and largely exceeded the values found in the other infants homozygous for F508del (see the original tracings in supplementary material S6). In studies using older patient groups, such high ICMs have not been reported using perfused systems [44–46], and only rarely in patient samples using recirculating Ussing chambers [47]. The unexpectedly 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 or inflammatory components. Patient-specific mechanisms that control F508del protein maturation and apical trafficking, which are not maintained in the organoid culture, may

also contribute [46], as may the impact of cAMP/Ca^{2+}-stimulated signalling 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 relatively high number of ICM values were missing. Furthermore, organoid swelling is highly CFTR dependent, but is not a direct readout of CFTR function; it relies on coupling of CFTR-dependent ion transport to fluid transport. Although the assay is relatively straightforward and robust as compared to other *in vitro* readouts, as indicated by nine 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 centres for centralised and standardised 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 outcomes 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 the FIS assay can play a role in determining 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 by FIS of intestinal organoids identified subgroups that differed in pulmonary and gastrointestinal clinical outcome parameters. FIS correlated well with the 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 the idea 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.

Conflict of interest: J.M. Beekman reports a licensed patent, number CA2859614 A1, for a rapid quantitative assay to measure CFTR function in a primary intestinal culture model. J.F. Dekkers reports a licensed patent, number CA2859614 A1, for a rapid quantitative assay to measure CFTR function in a primary intestinal culture model. R. Vries reports support from Vertex for drug screening and from CZ for development of diagnostics, outside the submitted work. H.A.W.M. Tiddens reports fees/grants from Roche for an industry symposium on treatment in CF, from Novartis for lectures and advisory boards, from CFF for Lung Analysis, from Vertex for Lung Analysis and advisory boards, from Gilead for lectures and advisory boards, and from Chiesi for an investigator-initiated trial, all outside the submitted work. He also reports a licensed combined patent from Vectura on specific targeting with DNase, and an issued patent from the PRAGMA-CF scoring system. He heads the Erasmus Medical Center/Sophia Children's Hospital core laboratory Lung Analysis. K.M. de Winter-de Groot has nothing to disclose. H.M. Janssens has nothing to disclose. R.T. van Uum has nothing to disclose. G. Berkers has nothing to disclose. A. Vonk has nothing to disclose. E. Kruisselbrink has nothing to disclose. H. Oppelaar has nothing to disclose. H. Clevers has nothing to disclose. R.H.J. Houwen has nothing to disclose. J.C. Escher has nothing to disclose. S.G. Elias has nothing to disclose. H.R. de Jonge has nothing to disclose. Y.B. de Rijke has nothing to disclose. C.K. van der Ent has nothing to disclose.

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