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Danya Muilwijk[1,15], Eyleen de Poel[1,2,15], Peter van Mourik[1], Sylvia W.F. Suen[1,2], Annelotte M. Vonk[1,2], Jesse E. Brunsveld[1,2], Evelien Kruisselbrink[1,2], Hugo Oppelaar[1,2], Marne C. Hagemeijer[1,2,3], Gitte Berkers[1], Karin M. de Winter-de Groot[1], Sabine Heida-Michel[1], Stephan R. Jans[1], Hannah van Panhuis[1], Menno M. van der Eerden[4], Renske van der Meer[5], Jolt Roukema[6], Edward Dompeling[7], Els J.M. Weersink[8], Gerard H. Koppelman[9,10], Robert Vries[11], Domenique D. Zomer-van Ommen[12], Marinus J.C. Eijkemans[13], Cornelis K. van der Ent[1,16] and Jeffrey M. Beekman[1,2,14,16]

[1]Dept of Pediatric Respiratory Medicine, Wilhelmina Children's Hospital, University Medical Center, Utrecht University, Utrecht, The Netherlands. [2]Regenerative Medicine Utrecht, University Medical Center, Utrecht University, Utrecht, The Netherlands. [3]Center for Lysosomal and Metabolic Diseases, Dept of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands. [4]Dept of Pulmonology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands. [5]Haga Teaching Hospital, The Hague, The Netherlands. [6]Radboud University Medical Center, Radboud Institute for Health Sciences, Nijmegen, The Netherlands. [7]Maastricht University Medical Center, Maastricht, The Netherlands. [8]Amsterdam University Medical Center, location AMC, Amsterdam, The Netherlands. [9]University of Groningen, University Medical Center Groningen, Beatrix Children's Hospital, Department of Pediatric Pulmonology and Pediatric Allergology, Groningen, The Netherlands. [10]University of Groningen, University Medical Center Groningen, Groningen Research Institute for Asthma and COPD (GRIAC), Groningen, The Netherlands. [11]Hubrecht Organoid Technology (HUB), Utrecht, The Netherlands. [12]Dutch Cystic Fibrosis Foundation (NCFS), Baarn, The Netherlands. [13]Dept of Data Science and Biostatistics, Julius Center for Health Sciences and Primary Care, University Medical Center, Utrecht University, Utrecht, The Netherlands. [14]Center for Living Technologies, University Medical Center, Utrecht, The Netherlands. [15]These authors contributed equally to this work. [16]C.K. van der Ent and J.M. Beekman contributed equally to this article as lead authors and supervised the work.

Corresponding author: Jeffrey M. Beekman (J.Beekman@umcutrecht.nl)

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Forskolin-induced swelling of patient-derived intestinal organoids is associated with long-term cystic fibrosis disease progression, expressed as FEV_1pp decline and development of pancreatic insufficiency, CF-related liver disease and CF-related diabetes https://bit.ly/3tjjJzU

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Abstract

Rationale Cystic fibrosis (CF) is a monogenic life-shortening disease associated with highly variable individual disease progression which is difficult to predict. Here we assessed the association of forskolin-induced swelling (FIS) of patient-derived organoids with long-term CF disease progression in multiple organs and compared FIS with the golden standard biomarker sweat chloride concentration (SCC).

Methods We retrieved 9-year longitudinal clinical data from the Dutch CF Registry of 173 people with mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Individual CFTR function was defined by FIS, measured as the relative size increase of intestinal organoids after stimulation with 0.8 µM forskolin, quantified as area under the curve (AUC). We used linear mixed-effect models and multivariable logistic regression to estimate the association of FIS with long-term forced expiratory volume in 1 s % predicted (FEV_1pp) decline and development of pancreatic insufficiency, CF-related liver disease and diabetes. Within these models, FIS was compared with SCC.

Results FIS was strongly associated with longitudinal changes of lung function, with an estimated difference in annual FEV_1pp decline of 0.32% (95% CI 0.11–0.54%; p=0.004) per 1000-point change in AUC. Moreover, increasing FIS levels were associated with lower odds of developing pancreatic insufficiency (adjusted OR 0.18, 95% CI 0.07–0.46; p<0.001), CF-related liver disease (adjusted OR 0.18, 95% CI 0.06–0.54; p=0.002) and diabetes (adjusted OR 0.34, 95% CI 0.12–0.97; p=0.044). These associations were absent for SCC.

Conclusion This study exemplifies the prognostic value of a patient-derived organoid-based biomarker within a clinical setting, which is especially important for people carrying rare *CFTR* mutations with unclear clinical consequences.

Introduction

Clinical disease expression in people with cystic fibrosis (CF) is variable and results from a combination of genetic, environmental and stochastic factors that are unique for each individual. CF is a recessive, monogenic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [1]. More than 2000 *CFTR* variants which differentially affect CFTR function and clinical phenotype have been identified (http://cftr2.org). The more common mutations have been categorised into distinct classes according to the mechanism by which CFTR function is disrupted [2]. To better understand how CFTR function contributes to disease expression, biomarkers such as sweat chloride concentration (SCC), intestinal current measurements (ICM) and nasal potential difference (NPD) are used to estimate individual CFTR function. These biomarkers have mostly been validated in the context of CF diagnosis, but their ability to accurately discriminate between people with CF with differential disease progression is limited despite clear relationships at population level [3–9]. Forskolin-induced swelling (FIS) of patient-derived intestinal organoids is an *in vitro* biomarker that quantifies CFTR-dependent fluid transport into the organoid lumen [10, 11] and may provide a more precise and accurate estimation of CFTR function compared to other biomarkers. Small proof-of-concept studies showed that FIS correlates with SCC and ICM and that clinical disease phenotypes could be stratified based on FIS level [12, 13]. We hypothesised that individual CFTR function measured by FIS is associated with long-term disease progression defined by rate of forced expiratory volume in 1 s % predicted (FEV_1pp) decline and development of comorbidities such as pancreatic insufficiency, CF-related liver disease and CF-related diabetes. Such an association supports a potential role for FIS as biomarker for long-term disease progression, which is especially relevant to people with rare, uncharacterised *CFTR* genotypes or *CFTR* genotypes with varying clinical consequences.

Methods

Study design and population

A longitudinal cohort study was conducted in Dutch people carrying mutations in the *CFTR* gene who are included in the Dutch Cystic Fibrosis Registry (DCFR). For all participants, intestinal organoids were generated before January 2020 and written informed consent was obtained to use their intestinal organoids and clinical data for the present study. This study was approved by the institutional review board of the University Medical Center Utrecht (Utrecht, the Netherlands).

Study parameters

The primary outcome variable was defined as long-term lung function decline, expressed as FEV_1pp, calculated according to Global Lung Function Initiative guidelines [14]. Secondary outcome variables were occurrence of pancreatic insufficiency, defined by faecal elastase <200 $\mu g \cdot g^{-1}$; CF-related liver disease, defined by hepatic steatosis or cirrhosis confirmed by imaging; and occurrence of insulin-dependent CF-related diabetes, defined by daily insulin treatment.

The primary explanatory variable of interest was FIS, defined by the relative size increase of intestinal organoids after 1 h stimulation with 0.8 µM forskolin, quantified as area under the curve (AUC). Previous studies showed that discrimination between individual FIS responses was most optimal and correlated best with other *in vitro* and *in vivo* CFTR biomarkers when FIS was performed with 0.8 µM forskolin [11, 12]. Other explanatory variables included were age (in years) at time of each lung function measurement; treatment status at time of each lung function measurement, categorised as no CFTR modulator treatment, treatment with ivacaftor or with lumacaftor/ivacaftor; sex; SCC in $mmol \cdot L^{-1}$; and genotype, categorised as class I–V or unclassified, defined by genotype class of the mildest of both mutations according to the available literature (supplementary tables S1 and S2). Additionally, genotypes were categorised in groups according to the combination of the following mutation types: insertion/deletion, nonsense, missense, splice and unknown.

Study procedures

Organoid measurements

The generation of intestinal organoids from biopsies and subsequent fluid secretion assays (FIS-assays) were performed according to a previously described protocol [15]. Rectal biopsies were collected at one time point during the 9-year study period. The specific time point of rectal biopsy collection varied per study participant, but was always prior to the start of modulator therapy. FIS-assays were performed between 2014 and 2020 by analysts who were blinded for genotype and clinical data. All FIS-assay

experiments were conducted in duplicate and for the majority of the donors at multiple culturing time points with a maximum of seven consecutive culture time points (n=7).

Clinical data collection

Data on clinical study parameters were retrieved from the DCFR, independent of FIS-assay results. Annual best FEV_1pp values between 2010 and 2018 were used to estimate lung function decline. Treatment status at the time of each lung function measurement was calculated based on start and stop dates of CFTR modulators as registered in the DCFR. For SCC, pancreatic insufficiency, CF-related liver disease and CF-related diabetes, we only collected the most recent value registered before 2019 (or before CFTR modulator treatment initiation, if applicable), as repeated measurements were unavailable or inconsistently collected. For SCC, pancreatic insufficiency, CF-related liver disease and CF-related diabetes, data were missing in 59 (34.1%), 63 (36.4%), five (2.9%) and three (1.7%) participants, respectively. SCC values were mostly missing for older participants, which may have been performed years before start of the registry in 2010 and were not archived within the local CF centres.

Statistical analysis

The association between age and long-term lung function decline was analysed using a linear mixed-effects model. FEV_1pp was specified as outcome variable in the model, with FIS, SCC, genotype class (reference category: unclassified), sex (reference category: male), age, CFTR modulator treatment (reference category: none) and FIS×age as fixed effects, where the interaction term FIS×age reflected the difference in annual FEV_1pp decline by FIS level. The model included a random intercept and random slope for age per subject, assuming a first-order auto-regressive (cAR1) correlation structure. Conditional R^2 was calculated to assess overall model performance and marginal R^2 to estimate the relative contribution of the fixed effects.

To account for selection bias towards a milder phenotype in participants surviving to an older age, a subgroup analysis was conducted including measurements performed between 4 and 25 years of age, in which the relationship between age and FEV_1pp decline can reasonably be assumed to be linear in this dataset (figure 2a).

Sensitivity analyses were performed using genotype group, defined by the combination of mutation types, *e.g.* insertion/deletion, nonsense, missense, splice, unknown. Genotype group was used instead of genotype class, to assess whether the association of FIS with FEV_1pp decline was influenced by categorisation of genotype. To obtain reliable effect estimates and standard errors for genotype group, groups with fewer than five participants were excluded from this part of the analysis.

To compare the association of long-term FEV_1pp decline with FIS *versus* SCC, four models were built which all included FIS, SCC, genotype class, sex, age and treatment as fixed effects. A baseline model was built without any interaction term, and the other three models were built with the addition of either the interaction term FIS×age, SCC×age or both FIS×age and SCC×age in the model. Performance of these models was compared using the likelihood ratio test.

Multilevel multiple imputation based on the method of chained equations [16] was used to handle missing SCC data in the linear mixed-effects models. All analyses were performed on 20 imputed datasets (m=20, iterations=20) with pooling of the results.

Secondary outcomes were analysed using multivariable logistic regression, with FIS, SCC, sex and age at the last study measurement as explanatory variables. Given the low proportion of outcome events within some of the genotype classes as well as within genotype groups (defined by the combination of the mutation types on both alleles), genotype could not be included in these analyses. In addition, CFTR modulator usage was not included, as we only collected most recent values of pancreatic insufficiency, CF-related liver disease and CF-related diabetes before modulator initiation. Nagelkerke's R^2 was calculated to assess model performance.

Single-level multiple imputation [16] was used to handle missing data of SCC, pancreatic insufficiency and CF-related diabetes in the logistic regression models. The analyses were performed on 20 imputed datasets (m=20, iterations=20) with pooling of the results.

Significance levels were set at 0.05. All statistical analyses were performed with R version 4.1.1 using packages mice, micemd, nlme and lme4 in combination with the performance package.

Results

Participant characteristics

In total, 173 participants carrying different *CFTR* genotypes provided written informed consent to collect intestinal organoid data and retrieve their clinical data from the DCFR. Participant characteristics are summarised in table 1. Three participants were excluded from the analysis because clinical data were not available. No data were excluded based on organoid measurements. Classification per mutation, individual genotypes with corresponding mutation classification and mutation group are listed in supplementary tables S1 and S2, respectively.

Individual FIS responses

Individual FIS responses after 1 h of stimulation with different forskolin concentrations are shown for all participants in figure 1a. Between-subject variability was most apparent at 0.8 µM and 5.0 µM forskolin, but no evident clustering was observed. Consistent with prior studies investigating relations between FIS and CF disease or biomarkers [11, 12, 17], our analyses were performed with FIS levels upon 0.8 µM forskolin stimulation. FIS data at 0.8 µM forskolin was skewed and highly variable among participants (median, interquartile range (IQR) AUC 141.3, 30.3–1176.3; range −268.0–4508.8; figure 1a and supplementary figure S1a) as well as within genotype classes (figure 1b,c) and between genotype groups, defined by the combination of the two mutation types (supplementary figure S1b). As expected, most organoid cultures that showed residual CFTR function (AUC >750) expressed genotypes belonging to classes III–V (figure 1c). Surprisingly, seven organoid cultures expressing genotypes categorised as class II mutation, a class for which no residual organoid swelling upon stimulation with 0.8 µM for 1 h has been reported previously [11–13], exhibited moderate to high organoid swelling (figure 1b).

Association of long-term FEV_1pp decline and FIS

1054 observations of 149 participants with available FEV_1pp measurements (figure 2a) were included in the analysis to assess the association of FIS with long-term FEV_1pp decline. Linear mixed-model analysis

TABLE 1 Participant characteristics

Participants	173
Age (years)	19.5 (9.5–30.5)
Sex	
Male	87 (50.3)
Female	86 (49.7)
Mutation class[#]	
Class I	15 (8.7)
Class II	91 (52.5)
Class III	11 (6.4)
Class IV	10 (5.8)
Class V	23 (13.3)
Unclassified	23 (13.3)
CFTR modulator usage	
Ivacaftor	16 (9.2)
Lumacaftor/ivacaftor	8 (4.6)
FIS[¶]	141.3 (30.3–1176.3)
SCC (mmol·L^{-1})	92.6±25.9
Missing values	59 (34.1)
FEV_1pp	75.9±23.2
Pancreatic function	
Insufficient (faecal elastase <200 µg·g^{-1})	75 (43.4)
Sufficient (faecal elastase ⩾200 µg·g^{-1})	35 (20.2)
Missing values	63 (36.4)
CF-related liver disease	44 (25.4)
Missing values	5 (2.9)
CF-related diabetes	25 (14.5)
Missing values	3 (1.7)

Data are presented as n, median (interquartile range), n (%) or mean±SD. CFTR: cystic fibrosis transmembrane conductance regulator; FIS: forskolin-induced swelling; SCC: sweat chloride concentration; FEV_1pp: forced expiratory volume in 1 s, % predicted; CF: cystic fibrosis. [#]: genotype class of the mildest of both mutations; [¶]: defined as the relative size increase of intestinal organoids (area under the curve) after 1 h stimulation with 0.8 µmol·L^{-1} forskolin.

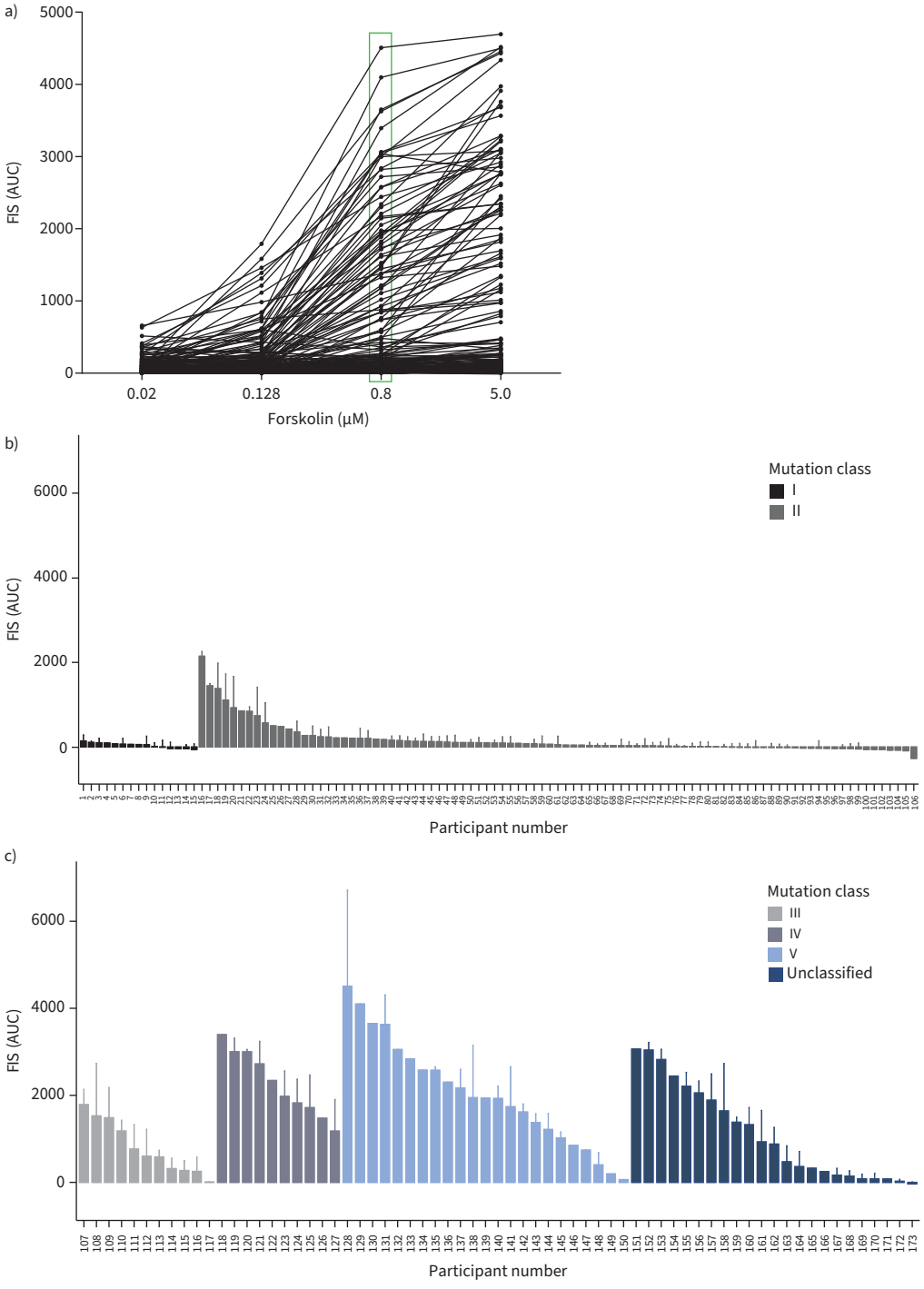

FIGURE 1 Forskolin-induced swelling (FIS) levels of organoids derived from the 173 study participants. a) FIS levels, defined by relative size increase of intestinal organoids after 1 h stimulation with four ascending forskolin concentrations, quantified as area under the curve (AUC). Each line represents swelling of organoids of individual study participants. Each data point represents mean AUC of both technical (n=2) and biological replicates (ranging from n=1 to n=7). b and c) Waterfall plots of FIS responses at 0.8 µM forskolin (highlighted by the green box in a)) of all study participants grouped based on b) mutation class I or II or c) mutation classes III–V or unclassified. Genotypes are categorised into one mutation class based on the mildest mutation class of the two alleles. Bars represent mean+SD of all replicates. Corresponding genotypes for the numbered participants are specified in supplementary table S2.

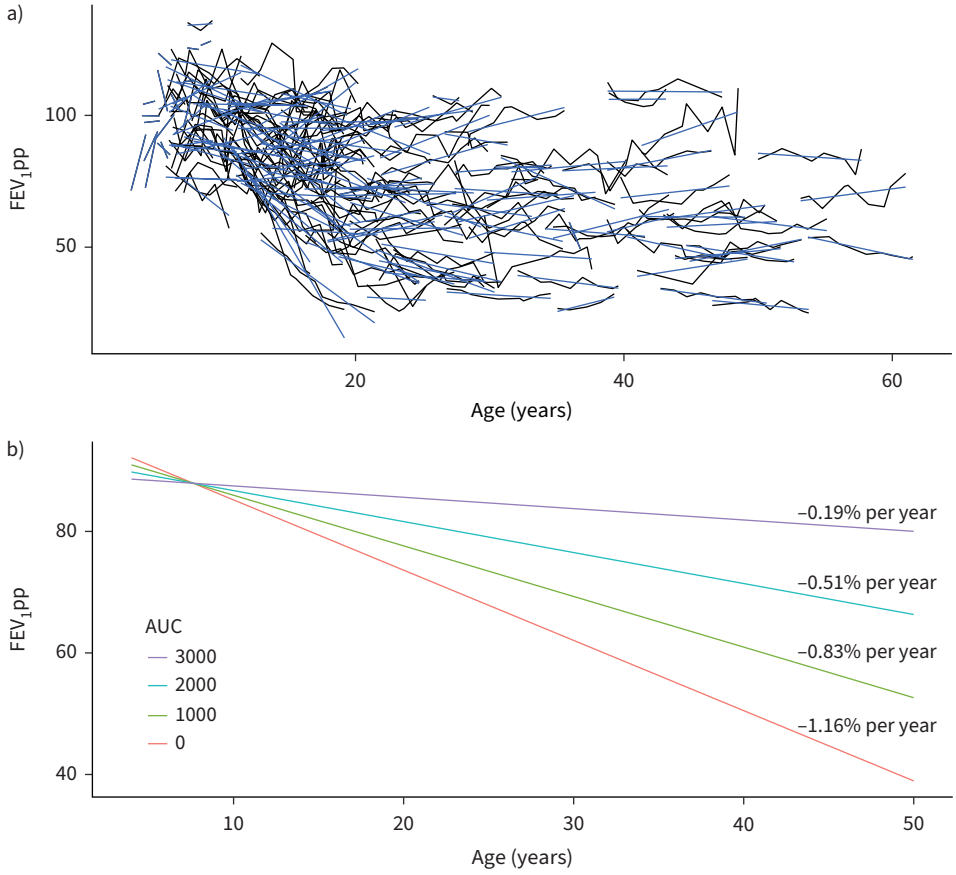

FIGURE 2 Association of forskolin-induced swelling (FIS) with long-term forced expiratory volume in 1 s % predicted (FEV_1pp) decline. a) Individual FEV_1pp trajectories of study participants over time in years. Black lines represent individual observed FEV_1pp trajectories, whereas the blue lines represent estimated average annual FEV_1pp slope per individual. b) Predicted FEV_1pp decline based on linear mixed-effects model coefficients in table 2, illustrating the association between different levels of residual cystic fibrosis transmembrane conductance regulator (CFTR) function and long-term FEV_1pp decline. Analysis was performed with FIS as a continuous variable, yet for illustrative purposes predicted FEV_1pp decline is plotted by steps of 1000-point change in area under the curve (AUC). Average predicted annual FEV_1pp decline per 1000 AUC is specified on the right. The lower limit of the x-axis was set at 4 years, because the feasibility and generalisability of FEV_1pp measurements is limited for younger children. Pooled conditional R^2=0.977, marginal R^2=0.179.

showed that average FEV_1pp decline per year of age varied with FIS level, adjusted for sex, genotype class, CFTR modulator usage and SCC (table 2). To illustrate this association of FEV_1pp decline by age with FIS, figure 2b shows that average annual FEV_1pp decline was −1.16% (95% CI −1.43%– −0.88%; p<0.001) per year of age for participants with a FIS level of 0. Per 1000-point increase in AUC, FEV_1pp decline was 0.32% (95% CI 0.11–0.54%; p=0.004) per year of age lower, leading to a very mild estimated FEV_1pp decline of only −0.19% per year for participants with an AUC of 3000. Model performance was excellent based on a pooled conditional R^2 of 0.979 (pooled marginal R^2=0.179).

The validity of these results was verified by assessing the potential impact of selection bias and confounding with separate subgroup and sensitivity analyses. A subgroup analysis in participants aged between 4 and 25 years showed a slightly higher average annual FEV_1pp decline compared to the complete population (−1.57% per year, 95% CI −2.03– −1.10%; p<0.001). Similar to the analysis in the complete cohort, FEV_1pp decline varied by FIS level with 0.49% (95% CI 0.03–0.96%; p=0.039; supplementary table S3 and supplementary figure S2) per 1000-point change in AUC, suggesting a negligible impact of selection bias due to the inclusion of people with *CFTR* mutations who have a milder phenotype and survive to an older age. Since at least one *CFTR* mutation was unclassified in 13.3% of participants (figure 1c, table 1 and supplementary tables S1 and S2), a sensitivity analysis was performed in which we refitted both models with genotype group instead of genotype class, to assess whether the

TABLE 2 Association of forskolin-induced swelling (FIS)[#] with forced expiratory volume in 1 s % predicted (FEV_1pp) decline

	Coefficient (95% CI)	p-value
Age	−1.16 (−1.43– −0.88)	<0.001*
FIS	−2.47 (−8.92–3.99)	0.454
FIS×age[¶]	0.32 (0.11–0.54)	0.004*
Treatment		
None	Reference category	
Ivacaftor	7.99 (4.58–11.40)	<0.001*
Lumacaftor/ivacaftor	−3.83 (−8.28– −0.62)	0.092
Sex		
Male	Reference category	
Female	−0.96 (−7.00–5.08)	0.754
Genotype class[+]		
Unclassified	Reference category	
Class I	0.18 (−13.92–14.27)	0.980
Class II	5.13 (−5.76–16.01)	0.356
Class III	10.25 (−3.79–24.28)	0.152
Class IV	11.01 (−5.36–27.38)	0.187
Class V	−2.31 (−16.95–12.33)	0.757
SCC	−0.09 (−0.25–0.06)	0.239

Regression coefficients of linear mixed-effects model for FEV_1pp. n=149, n=1054 observations. SCC: sweat chloride concentration. [#]: defined as the relative size increase of intestinal organoids (area under the curve (AUC)) after 1 h stimulation with 0.8 μM·L^{-1} forskolin, coefficient scaled 1:1000 AUC; [¶]: indicates the difference in annual FEV_1pp decline per 1000 AUC change in FIS level; [+]: cystic fibrosis transmembrane conductance regulator (CFTR) protein function class of the mildest of both CFTR mutations. Pooled conditional R^2=0.979, marginal R^2=0.179. *: p<0.05.

association of FIS with FEV_1pp decline was influenced by categorisation of genotype. The association of FIS with FEV_1pp decline in these models was still statistically significant, comparable to the models categorising genotype by mutation class (supplementary table S4).

In addition, we compared the association of FIS with FEV_1pp decline *versus* SCC with FEV_1pp decline in similar linear mixed models. SCC alone was not significantly associated with FEV_1pp decline (p=0.121; supplementary table S5). An association with SCC was also absent (p=0.995; supplementary table S6) when combined with FIS in the model, suggesting a stronger association of FIS with FEV_1pp decline compared to SCC. However, these results should be interpreted with caution due to the proportion of missing SCC data and the use of multiple imputation.

Association of CF-related comorbidities and FIS

To investigate the association of FIS with the occurrence of other CF-related comorbidities, we performed multivariable logistic regression with pancreatic insufficiency, CF-related liver disease and CF-related diabetes, adjusted for age, sex and SCC. We found a significant association of FIS with the occurrence of pancreatic insufficiency (adjusted OR 0.18, 95% CI 0.07–0.46; p<0.001, Nagelkerke's R^2=0.496), CF-related liver disease (adjusted OR 0.18, 95% CI 0.06–0.54; p=0.002, Nagelkerke's R^2=0.222) and CF-related diabetes (adjusted OR 0.34, 95% CI 0.12–0.97; p=0.044, Nagelkerke's R^2=0.195; table 3 and figure 3a–d). This indicates that the odds were on average five-fold lower for developing pancreatic insufficiency and CF-related liver disease and three-fold lower for developing CF-related diabetes per 1000-point increase in FIS level. As illustrated in table 3 and figure 3d, age was also significantly associated with the odds of developing CF-related diabetes (adjusted OR 1.05, 95% CI 1.02–1.08; p=0.004).

In combination with FIS, SCC was not associated with any of the CF-related comorbidities, given the nonsignificant OR of 1 (table 3). Even though multiple imputation of SCC may have influenced the strength of the associations, these results suggest that FIS is more strongly associated with CF-related comorbidities than SCC when comparing both biomarkers within the same model.

Discussion

This study shows that residual CFTR function quantified by FIS of patient-derived CF organoids is associated with long-term annual FEV_1pp decline and odds of developing the CF-related comorbidities

TABLE 3 Association of forskolin-induced swelling (FIS)[#] with cystic fibrosis (CF)-related comorbidities

	Pancreatic insufficiency		CF-related liver disease		CF-related diabetes	
	Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value
FIS	0.18 (0.07–0.46)	<0.001*	0.18 (0.06–0.54)	0.002*	0.34 (0.12–0.97)	0.044*
Age	0.98 (0.93–1.02)	0.300	1.02 (0.99–1.05)	0.229	1.05 (1.02–1.08)	0.004*
Sex						
Male	Reference category	0.181	Reference category	0.313	Reference category	
Female	0.46 (0.14–1.46)		0.68 (0.32–1.44)		2.08 (0.81–5.37)	0.127
SCC	1.00 (0.97–1.04)	0.944	1.00 (0.98–1.02)	0.913	1.00 (0.97–1.04)	0.838

Adjusted odds ratios of multivariable logistic regression for pancreatic insufficiency, CF-related diabetes and CF-related liver disease. n=170. SCC: sweat chloride concentration. [#]: defined as the relative size increase of intestinal organoids (area under the curve (AUC)) after 1 h stimulation with 0.8 $\mu M \cdot L^{-1}$ forskolin, coefficient scaled 1:1000 AUC; Nagelkerke's R^2 pancreatic insufficiency=0.496, CF-related liver disease=0.223, CF-related diabetes=0.195. *: p<0.05.

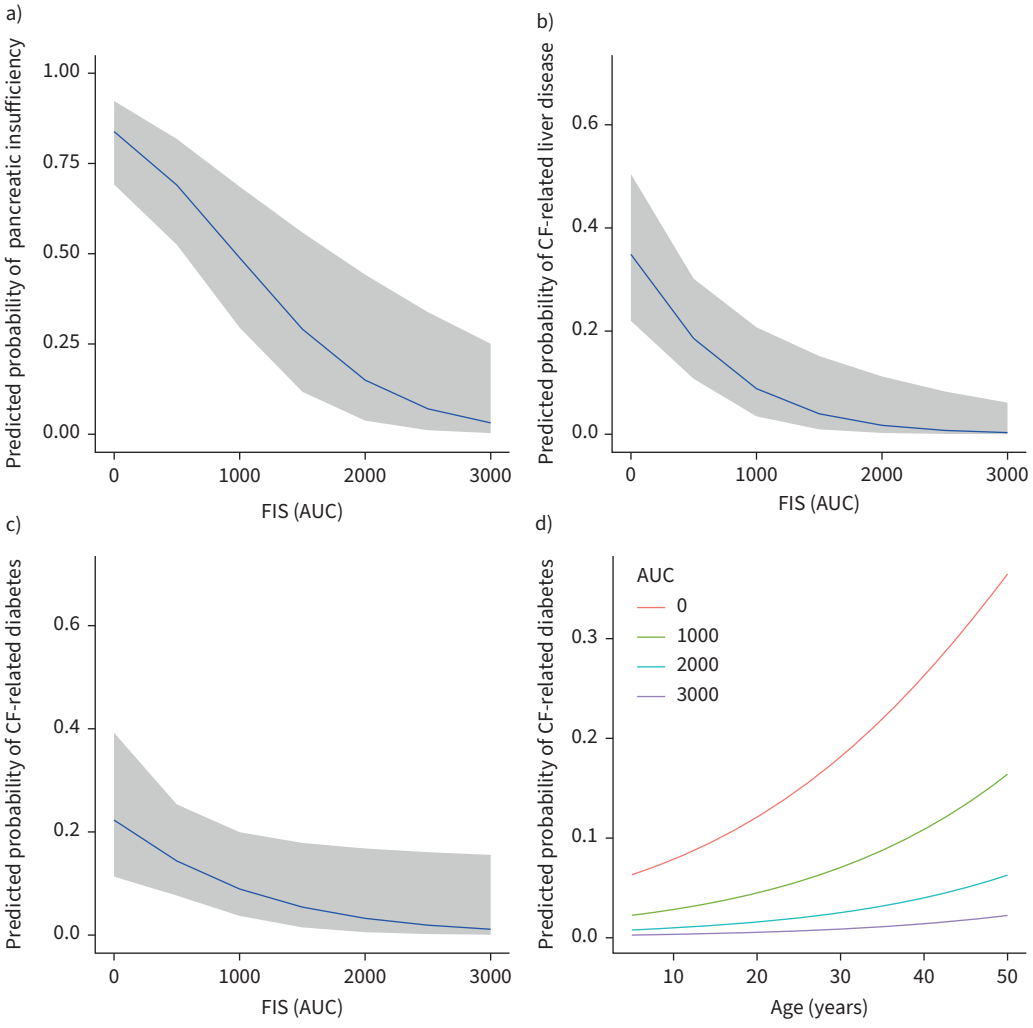

FIGURE 3 Association of forskolin-induced swelling (FIS) with cystic fibrosis (CF)-related comorbidities. Association between residual cystic fibrosis transmembrane conductance regulator function (illustrated by steps of 1000-point change in area under the curve (AUC)) and odds of developing a) pancreatic insufficiency, b) CF-related liver disease and c) CF-related diabetes. d) In addition to FIS, age is associated with the odds of developing CF-related diabetes. Nagelkerke's R^2: pancreatic insufficiency=0.496, CF-related liver disease=0.223, CF-related diabetes=0.195.

pancreatic insufficiency, CF-related liver disease and CF-related diabetes, using 9-year longitudinal data of Dutch people with many distinct *CFTR* mutations and ages ranging from 0 to 61 years.

Despite the influence of genetic modifiers and other non-CFTR-dependent environmental factors on CF disease severity [1, 18–20], it was remarkable to observe that *in vitro* FIS of intestinal cells has such a broad association with many nonintestinal organ systems. It illustrates that fluid secretion properties of CFTR in intestinal organoids are reflective of or related to CFTR function across many tissues.

As this study aimed to characterise *in vitro* CFTR function of many different common and rare *CFTR* mutations with FIS, the distribution of genotypes in our dataset does not correspond to the distribution of genotypes typical for the Dutch population, in which the F508del/F508del is the most common genotype. Yet it improves the generalisability of our results to the population with rare *CFTR* mutations, for which this study is especially relevant. In addition, rectal biopsies of the participants that have received modulator therapy were collected prior to the start of modulator therapy, so intestinal organoid measurements were not influenced by treatment.

Direct comparison of FIS with SCC revealed that FIS was more strongly associated with long-term multiorgan disease expression compared to SCC, which has been the most important and well-validated biomarker of CF disease until now and is a commonly used end-point to measure efficacy of CFTR-modulating drugs [5, 6]. Although the association with SCC could have been influenced by missing values and type of imputation method, the difference between FIS and SCC might also be explained by a more precise and accurate estimation of CFTR function by FIS. FIS facilitates repeated measures and is completely CFTR dependent, which reduces the impact of technological and non-CFTR biological variability in the *in vitro* assay [10, 11], whereas a substantial part of variability in SCC is caused by technical and other non-CFTR-dependent biological factors [5]. Additional studies with complete datasets including repeated measurements for more precise typing of SCC are required to confirm these findings. Alternatively, it would be interesting to explore if novel sweat-based readouts that may show a higher dependency on CFTR function might also lead to better correlations with clinical observations.

In addition, FIS could be compared with other biomarkers that are being used for CF diagnosis, such as NPD and ICM. Although NPD has been used to discriminate between non-CF and CF [3, 4, 6–9], its ability to discriminate accurately between people with CF with differential disease progression is limited. While ICM measurements are more sensitive and have a larger dynamic range than NPD, the generation of a large dataset with repeated measures is hampered by the need for fresh rectal biopsies.

Furthermore, the data suggested that FIS has additional value in the context of disease severity association beyond the current CFTR mutation classification system. For our statistical models, we needed to prioritise one particular mutational subclass for each CFTR mutation, which is difficult due to lack of detailed experimental data for many rare (missense) mutations and the impact of potential multiple mechanistic defects for single mutations [21]. This complicates studies of mutation classification and relationship with disease severity. CFTR function by FIS demonstrated a large variability in CFTR function between participants with different genotypes, but also within genotype classes. Thus, FIS may have the potential to help to further refine patient-based classification systems beyond current genotype classification models. This might lead to more precise individual typing and prediction of disease, compared to the current classification of “mild” and “severe” CF phenotypes [22–24] or the CFTR2-based classification of mutations (CF-causing, varying clinical consequences, non-CF causing).

Rates of annual FEV_1pp decline in this study were within the same range as reported by other recent European studies, which also showed that annual FEV_1pp decline was lower for people with CF with a “milder” disease severity as classified by genotype [25] or pancreatic status [26] and was highest in the age group between 18 and 28 years [26]. Moreover, our results are consistent with a previous study showing a more severe CF disease phenotype in terms of pulmonary and gastrointestinal outcome parameters in infants with low FIS compared to infants with high FIS [12]. In line with our observations, Davis *et al.* [27] demonstrated that SCC by itself does not predict lung disease in people with CF.

In addition to the relationship of FIS with disease severity, several studies have shown that average FIS response to CFTR modulators correlates with short-term clinical drug response across groups with different genotypes [11, 17] and in individuals with a variety of CFTR mutations [28]. Different exploratory studies did not detect an association of FIS with short-term clinical response to lumacaftor/ivacaftor in people with CF homozygous for F508del [29] or heterozygous for the A455E mutation [30] or to ivacaftor in people with residual CFTR-function mutations [31]. These studies did not demonstrate associations between FIS

and biomarkers of CFTR function (NPD, SCC and ICM) [29] or FIS and SCC [30, 31], nor relationships between any biomarker of CFTR function and clinical response. Additionally, treatment magnitude at group level was absent [29, 30] or limited [31], suggesting that the relative impact of CFTR-dependent factors over non-CFTR-dependent factors to between-patient variations was lower as compared to the study of Berkers *et al.* [28]. This generally lowers the ability of FIS or any individual outcome to correlate after a CFTR modulator treatment. Further research in larger study populations is therefore warranted to study the association of changes in FIS or other biomarkers of CFTR function with long-term clinical effects upon CFTR modulator therapy in homogeneous and heterogeneous populations with CF.

An important limitation of this research is the retrospective observational study design. We adjusted for several confounders, but were unable to account for other prognostic factors such as pulmonary exacerbations and sputum cultures. As 34% of SCC values was missing, we used multiple imputation methods to prevent bias due to selective missing data, but this may still have influenced the associations with SCC and its comparison with FIS. Potential impact of survival bias was minimised by our subgroup and sensitivity analyses, but could not be excluded completely. Additional prospective studies should be performed to confirm the predictive value of FIS in comparison with other biomarkers such as SCC, NPD and ICM, yet our findings are in line with previous work that already demonstrated the potential of FIS as biomarker of CF disease.

In summary, this study showed that FIS of cystic fibrosis intestinal organoids is strongly associated with long-term FEV_1pp decline and odds of developing different CF-related comorbidities, suggesting that estimation of CFTR function by FIS could have important prognostic value for individual disease expression of multiple, critical organs that are affected by CF.

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Conflict of interest: J.M. Beekman reports personal fees from Vertex Pharmaceuticals, Proteostasis Therapeutics, Eloxx Pharmaceuticals, Teva Pharmaceutical Industries and Galapagos, outside the submitted work; in addition, J.M. Beekman has a patent related to the FIS-assay with royalties paid. C.K. van der Ent reports grants from GSK, Nutricia, TEVA, Gilead, Vertex, ProQR, Proteostasis, Galapagos NV and Eloxx, outside the submitted work; in addition, C.K. van der Ent has a patent 10006904 with royalties paid. G.H. Koppelman reports grants from Lung Foundation of the Netherlands, Vertex Pharmaceuticals, UBBO EMMIUS foundation, GSK, TEVA the Netherlands, TETRI Foundation and European Union (H2020), outside the submitted work; and has participated in advisory board meetings for GSK and PURE-IMS outside the submitted work (money paid to institution). P. van Mourik reports financial compensation (money to institution) from Vertex for participation in a webinar, outside the submitted work. All other authors have nothing to disclose.

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