Rectal Organoids Enable Personalized Treatment of Cystic Fibrosis

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Graphical Abstract

Highlights
- Organoids of CF patients were used to quantitate individual drug response in vitro
- Organoid responses correlate with two clinical response parameters ppFEV₁ and SCC
- In vivo (non)responders were identified with a PPV of 100% and a NPV of 80%
- Organoids may be used for personalized medicine in cystic fibrosis

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In Brief
Berkers et al. demonstrate that stem cell cultures (organoids) can be a tool for personalized medicine. They show a high correlation between in vitro and in vivo effects of drugs and demonstrate good-to-excellent predictive values of the organoid test for preclinical identification of responders to CFTR modulators.
Rectal Organoids Enable Personalized Treatment of Cystic Fibrosis

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SUMMARY

In vitro drug tests using patient-derived stem cell cultures offer opportunities to individually select efficacious treatments. Here, we provide a study that demonstrates that in vitro drug responses in rectal organoids from individual patients with cystic fibrosis (CF) correlate with changes in two in vivo therapeutic endpoints. We measured individual in vitro efficaciousness using a functional assay in rectum-derived organoids based on forskolin-induced swelling and studied the correlation with in vivo effects. The in vitro organoid responses correlated with both change in pulmonary response and change in sweat chloride concentration. Receiver operating characteristic curves indicated good-to-excellent accuracy of the organoid-based test for defining clinical responses. This study indicates that an in vitro assay using stem cell cultures can prospectively select efficacious treatments for patients and suggests that biobanked stem cell resources can be used to tailor individual treatments in a cost-effective and patient-friendly manner.

INTRODUCTION

Functional drug testing on cells or tissue cultures of patients may represent a major step forward for selecting efficacious treatments in an individual setting. Our identification of Lgr5 as a marker of crypt stem cells and the development of technology to grow functional epithelial organoids from such stem cells allows the generation of disease- and patient-specific living biobanks (Barker et al., 2007; Sato et al., 2009; van de Wetering et al., 2015). These biobanks could serve as important resources for drug development and scientific studies, but examples demonstrating the validity of these tissue resources for the individual prediction of clinical drug efficacy are currently lacking.

Cystic fibrosis (CF) is a genetic disease that is caused by mutations of the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which leads to impaired protein function (Riordan et al., 1989). Over 2,000 CFTR mutations have been identified (http://www.genet.sickkids.on.ca/) and are associated with a variety of...
clinical phenotypes (https://www.cftr2.org/) (Sosnay et al., 2013; Cutting, 2015). Recently developed drugs for CF aim to restore CFTR protein function. Lumacaftor (VX-809) and tezacaftor (VX-661) are corrector drugs, influencing trafficking of the CFTR protein to the apical membrane, while ivacaftor (VX-770) is a potentiator drug, improving the function of the CFTR protein that is present at the apical membrane. In previous work, we showed that also the natural food components genistein and curcumin have potentiator activity in vitro, albeit at reduced efficacy and potency as compared to ivacaftor (Dekkers et al., 2016b). Currently, three CFTR-modulating drugs are registered for the treatment of CF patients with specific CFTR mutations: ivacaftor (VX770; Kalydeco) for patients with different CFTR gating mutations and patients with an R117H mutation, and a combination of ivacaftor and the CFTR correctors lumacaftor or tezacaftor (respectively, VX770+VX809, Orkambi, and VX770+VX661, Symdeco/Symkevi) for patients homozygous for the F508del mutation and some mutations associated with residual function in the case of Symdeco/Symkevi treatment (Ramsey et al., 2011; De Boeck et al., 2014; Moss et al., 2015; Wainwright et al., 2015; Rowe et al., 2017; Taylor-Cousar et al., 2017).

This CFTR genotype-based stratification for drug prescription presents a challenge for the inclusion of many people with rare CFTR mutations who are not included into clinical trials due to low prevalence of the mutation and lack of mechanistic insights. A recent label extension of ivacaftor by the US Food and Drug Administration (FDA), based on A recent label extension of ivacaftor by the US Food and Drug Administration (FDA), based on a recent meta-study found a small correlation between SCC and SCC suggested more precise quantification of CFTR function by FIS (de Winter-de Groot et al., 2018). We here describe the correlation between the response of FIS of rectal organoids and the in vivo therapeutic response for individual CF patients with multiple CFTR genotypes who were treated with several CFTR-modulating drugs, and we study the predictive values of the organoid FIS test for the clinical response.

RESULTS

To evaluate the relation between drug response in in vitro cultured organoids and therapeutic effect in vivo, we studied 37 paired in vitro-in vivo responses to three CFTR-modulating treatments in 24 subjects with CF (baseline characteristics are provided in Table 1). Fifteen patients with the ivacaftor-responsive S1251N mutation received ivacaftor (De Boeck et al., 2014). Thirteen of these patients first received a combination of the possible CFTR-potentiating food supplements genistein and curcumin before receiving ivacaftor (Dekkers et al., 2016b). The other nine patients carried at least one rare CFTR mutation with unknown clinical response and were selected for off-label treatment based on the organoid response to either ivacaftor or ivacaftor plus lumacaftor. Apart from the CFTR genotype, there were no relevant differences in the baseline clinical characteristics (such as percentage of predicted forced expiratory volume in 1 s [ppFEV1] or SCC values) between patients that received one or two treatments.

We quantified CFTR modulator responses in vitro by assessment of FIS of patient-derived rectal organoids that were previously cultured and stored in a biobank (Figures 1A and 1B show an example; individual measurements for all patients are provided in Figure S1). Organoid swelling was assessed after adding various concentrations of forskolin to facilitate optimal detection of drug response across the cohort for the various drugs (Dekkers et al., 2013). We used two outcome parameters to evaluate the in vivo clinical effect of a treatment: change in ppFEV1 and change in SCC. Pearson’s correlations between organoid response and pulmonary response were analyzed in a subgroup of patients who had a ppFEV1 ≥ 40% and ≤ 90% before the start of treatment, to limit non-response of this endpoint (ceiling effects at ≥90% or irreversible lung damage at <40%), as is usual in clinical trials (Ramsey et al., 2011; De Boeck et al., 2014; Moss et al., 2015; Wainwright et al., 2015; Wood et al., 2013; Taylor-Cousar et al., 2018). The organoid FIS positively correlated with both the pulmonary response (change in ppFEV1; \( n = 21, r = 0.610, p = 0.003 \); Figure 1C) and the change in SCC (\( n = 18, r = –0.762, p \leq 0.001 \); Figure 1D). As observed in other studies with CFTR modulators, the two in vivo endpoints appeared only weakly correlated, in a statistically non-significant manner (SCC versus ppFEV1,
n = 18, r = −0.366, p = 0.14; Figure 1E). We observed no big impact on the correlation of the repeated genistein plus curcumin and ivacaftor measurements; for ppFEV₁, n = 21, r = 0.624, p < 0.001, and for SCC, n = 18, r = −0.716, p < 0.001 (Figure S2). In accordance with previous observations, all correlations were optimal when organoid responses at 0.128 μM forskolin were used (Table S1) (Dekkers et al., 2016a). Patients with a ppFEV₁ >90% or ppFEV₁ <40% before the start of the treatment did not show a clear correlation between the organoid response and change in ppFEV₁, despite an identical correlation between organoids and SCC (Figures 1F and 1G). The data of all patients combined showed correlations of organoids with both ppFEV₁ (n = 35, r = 0.575, p < 0.001; Figure 1I) and SCC (n = 33, r = −0.708, p < 0.001; Figure 1J), but a statistically significant relation between ppFEV₁ and SCC was not observed (Figures 1H and 1K). People with rare mutations who were selected by organoids prior to treatment showed a median increase of 10% in ppFEV₁ (n = 7, p = 0.058) and a reduction of 39 mmol/L in SCC (n = 6, p = 0.028). Collectively, these data demonstrate that in vitro CFTR modulator responses in organoids correlate with two important therapeutic endpoints.

### Prediction of Clinical Responses Using Organoids

Next, we generated receiver operating characteristic (ROC) curves to examine the predictive potential of different organoid-based thresholds for identifying clinical responders. We dichotomized both the ppFEV₁ and SCC response into changes that are generally considered clinically significant and beyond the test variability (changes in ppFEV₁ >5%, or SCC >20 mM or a combined change in ppFEV₁ >5% and SCC >20 mM) and changes that are not (Seliger et al., 2013). The area under the ROC curve provides a general measure for test accuracy and was 0.837 (95% confidence interval [CI], 0.661–1.000) for predicting responders in ppFEV₁ and increased toward 0.938 (95% CI, 0.830–1.000) for predicting responders in either SCC or SCC and ppFEV₁ (Figure 2A). When repeated measurements were taken into account, the area under the ROC curve did not change. A Youden index was used to select an organoid cutoff point with the most optimal combination of sensitivity and specificity in an unbiased fashion (Youden, 1950). The selected cutoff value to identify responders in both SCC and ppFEV₁ had a sensitivity of 0.80 and a specificity of 1.00 with a corresponding Youden index of 0.8 for identifying responders and non-responders in both ppFEV₁ and SCC. The associated positive and negative predictive values were 100% and 80%, respectively. Since data-driven selection of the Youden index might cause over-estimation of both sensitivity and specificity, we performed a leave-one-out cross-validation to further validate our findings (Leeflang et al., 2008). This additional analysis showed a sensitivity of 0.70 and specificity of 1.00, with a corresponding Youden index of 0.70.

For patients that started with a ppFEV₁ <40% or >90%, the ROC curve had an area under the curve between 0.694 and 0.767 (Figure 2B). For the total group of patients that was treated,
Figure 1. Significant Correlation between Individual In Vitro Organoid Response and In Vivo Change in ppFEV₁ and SCC
(A) Confocal images of the forskolin-induced swelling (FIS) of organoids with an F508del/S1251N mutation. Images are taken 0 and 60 min after adding DMSO, genistein plus curcumin and ivacaftor (VX-770), in combination with forskolin.
(B) AUC of the swelling of organoids after measuring for 60 min. The graph shows responses after adding eight different concentrations of forskolin in combination with either DMSO or a CFTR-modulating treatment. Mean ± SD.
(C and D) Pearson correlations between response of the organoids of an individual patient upon CFTR-modulating treatment in combination with 0.128 mM forskolin and the in vivo response (change in ppFEV₁, as shown in C, and change in SCC, as shown in D) of the same patient to the same treatment for patients who had a ppFEV₁ ≥40% and ≤90% before the start of treatment.
(E) Pearson correlation between change in ppFEV₁ and change in SCC of individual patients upon a CFTR-modulating treatment for patients who had a ppFEV₁ <40% or >90% before the start of treatment.
(F and G) Pearson correlations between response of the organoids of an individual patient upon CFTR-modulating treatment in combination with 0.128 mM forskolin and the in vivo response (change in ppFEV₁, as shown in F, and change in SCC, as shown in G) of the same patient to the same treatment for patients who had a ppFEV₁ <40% or >90% before the start of treatment.
(H) Pearson correlation between change in ppFEV₁ and change in SCC of individual patients upon a CFTR-modulating treatment for patients who had a ppFEV₁ <40% or >90% before the start of treatment.

(legend continued on next page)
the area under the ROC curve varied between 0.783 and 0.869 (Figure 2C). Because of the small sample size, we did not calculate ROC curves for the group of patients that had at least one rare CFTR mutation.

In conclusion, the organoid-based test displayed excellent accuracy (area under the curve [AUC] of ROC curve, >0.9) for identifying clinical responses defined by changes in SCC and ppFEV1 or only SCC, while good accuracy (AUC of ROC curve, between 0.8 and 0.9) was observed for identifying clinical responses defined only by ppFEV1 (Metz, 1978).

**DISCUSSION**

This study aimed to provide evidence that FIS of rectal organoids can act as a prospective biomarker for *in vivo* CFTR modulator responses. We demonstrated here that individual *in vitro* CFTR modulator responses in these patient-derived stem cell cultures correlate with two independent indicators of therapeutic response *in vivo*. The moderate correlation between FIS and ppFEV1, and higher correlation between FIS and SCC (*in vivo* biomarker of CFTR function) is in agreement with the higher impact of non-CFTR-dependent factors on variation in pulmonary function as compared to SCC (Cutting, 2015; Collaco et al., 2016). We did not find a statistically significant correlation between change in SCC and ppFEV1, probably because of a weaker correlation between these outcome measurements in combination with a small sample size, as was previously also observed in other studies with comparable sample sizes (Accurso et al., 2010). These *in vivo* endpoints are suitable to indicate treatment effects at a group level, but non-CFTR-dependent variation in ppFEV1 and SCC probably limits their precision and accuracy for informing on individual CFTR function modulation (Fidler et al., 2016). In contrast, *in vitro* FIS is completely CFTR dependent and has sufficient sensitivity to quantitate CFTR modulator activity, and the repeated measurements increase precision. These properties likely facilitate that FIS has sufficient accuracy to inform on both ppFEV1 and SCC (or their combination), suggesting that FIS is a potent biomarker to quantitate individual CFTR modulator responses.

Our dataset provides a first analysis of the predictive potential of the rectal organoids to identify clinical responders and non-responders to treatment. Our data support that FIS can be used to prospectively select responders and non-responders to CFTR modulator treatments but the cutoff value with the highest Youden index still needs to be interpreted carefully as well as the definition of clinical responders. The Youden index selects the most optimal ratio between sensitivity and specificity, but a different threshold with a higher negative predictive value may be preferential to limit the exclusion of treatment responders (e.g., an organoid threshold with a negative predictive value of 100% would have a positive predictive value of 77%). Additionally, it remains unclear how short-term treatment responses individually translate into long-term clinical response. It could therefore be that the definitions for long-term clinical responders are different, leading to other threshold values of predictive tests. We observed that the correlation of the organoid test with response in ppFEV1 was modified by baseline ppFEV1, despite similar correlation in SCC in both groups with differences in baseline ppFEV1. This supports that biomarkers of CFTR function such as organoid-based measurements have an important role for assessment of CFTR modulator responses in subjects where clinical domain indicators are unsuited to measure therapeutic response.

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(i and J) Pearson correlations between response of the organoids of an individual patient upon CFTR-modulating treatment in combination with 0.128 μM forskolin and the *in vivo* response (change in ppFEV1, as shown in I, and change in SCC, as shown in J) of the same patient to the same treatment for all patients that received treatment.

(K) Pearson correlation between change in ppFEV1 and change in SCC of individual patients upon a CFTR-modulating treatment for all patients that received treatment.

See also Figures S1 and S2 and Table S1.
There are several limitations in this study. First, the open-label setting of treatments can induce bias in the acquisition of clinical data. Potentially, ppFEV₁ might have been influenced, but this is unlikely for SCC measurements. However, we do not expect that the open-label setting has strongly affected the in vitro-in vivo correlation, since the clinical observers and patients were blinded for the in vitro drug responses and vice versa. Second, the study is biased for potentiator treatments. The area under the ROC curves may be different when patients are stratified for different CFTR modulator treatments such as corrector/potentiator combinations. Also, the cutoff values of ppFEV₁ and SCC that were used to define a clinical responder may not be fully accurate in identifying long-term clinical responders to treatment, and changing these cutoff values will lead to different ROC curves. Third, patient subgroups with differences in organoid baseline CFTR functions may require different organoid test conditions (e.g., different forskolin conditions) for better predictive values. Fourth, it remains challenging to estimate adequate drug concentrations in the organoid tests as to optimally reflect the in vivo tissue concentration. For ivacaftor and lumacaftor, we relied on average blood concentrations to determine the in vitro drug concentrations (European Medicines Agency, 2018a, 2018b). For genistein and curcumin, lack of information on in vivo tissue concentrations may have resulted in overdosing the in vitro situation, which can lead to overestimation of their potential in vivo effect. Most importantly, larger follow-up studies remain needed to define more precisely how organoid-based measurements, and possibly other short-term endpoints, can predict long-term individual benefit to various CFTR modulator treatments. Apart from the performance of FIS as a biomarker of treatment response in this study, the rectal organoids provide additional benefits over other biomarkers of CFTR function. Rectal organoids are adult stem cell cultures that can be generated from a single rectal biopsy and cultured over 6 months while maintaining patient-specific CFTR modifier response (Clevers, 2016; Dekkers et al., 2016a). Rectal biopsies are accessible in most subjects independent of age and can be shipped to dedicated centers for organoid testing within weeks and stored in living biobanks, which enables future drug testing (Dekkers et al., 2016a). The FIS readout appears also not affected by CF disease phenotype (e.g., irreversible damage and inflammation in pulmonary markers). Currently, the immediate impact can be the selection of people for treatments independent of the CFTR genotype, both for CFTR modulators on the market and in development. For people having access to treatment, we may be able to further individually tailor treatments to maximize clinical benefits (Beekman, 2016).

**Conclusion**

In vitro drug efficacy measurements by FIS in rectal organoids of individuals with CF correlate with the most important in vivo response indicators of CFTR modulators (change in ppFEV₁ and SCC). The data further suggest that thresholds can be established to prospectively identify clinical responders with acceptable positive and negative predictive values. Organoid testing can provide a patient-friendly and cost-effective approach to increase access to treatment for patients with CF, and optimize risk-benefit and cost-effectiveness of treatments. This study is a first example that in vitro tests using cultures of patient stem cells, stored in living biobanks, can be used to predict individual treatment benefits.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes two figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2019.01.068.

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**AUTHOR CONTRIBUTIONS**

G.B. designed the clinical trials, provided clinical data, analyzed and interpreted results, and generated article text and figures. P.v.M. provided clinical data, interpreted results, and revised the manuscript. A.M.V., E.K., and J.F.D. were responsible for organoid cultures, performed the FIS experiments, and revised the manuscript. R.E.P.M.-v.d.W., J.S.D., and M.M.V. provided clinical data, interpreted results, and revised the manuscript. K.M.d.W.-d.G., H.G.M.A., R.H.J.H., H.G.M.H., E.A.v.d.G., C.J.M., G.H.K., J.R., M.B., H.M.J., and R.v.d.M. provided patient material and/or clinical data and revised the manuscript. S.G.E. analyzed and interpreted results and revised the manuscript. R.G.J.V. and H.C.C. designed experiments and revised the manuscript. H.R.d.j.d. designed the clinical trials and revised the manuscript. J.M.B. designed the organoid experiments, interpreted results, and generated article...
text. C.K.v.d.E. designed the clinical trials, provided patient material and clinical data, interpreted results, and generated article text.

DECLARATION OF INTERESTS

J.F.D., H.C.C., J.M.B., and C.K.v.d.E. are inventors on patent(s) related to these findings. G.H.K., J.M.B., and C.K.v.d.E. report receiving research grant(s) from Vertex Pharmaceuticals (money to institution) outside the submitted work.

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REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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<td><strong>Other</strong></td>
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<tr>
<td>Matrigel (protein concentration between 9.8-10.2 mg/ml)</td>
<td>Corning</td>
<td>Cat# 354230</td>
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</tbody>
</table>

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Jeffrey M. Beekman (j.beekman@umcutrecht.nl).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Forskolin induced swelling of rectal organoids

Rectal organoids were cultured according to previously described protocols, and are accessible for study by contacting the Huberecht Organoid Technology foundation (http://hub4organoids.eu) (Sato et al., 2011; Dekkers et al., 2016a). Forskolin-induced
swelling of rectal organoids is a fully CFTR-dependent readout and was measured to indicate baseline CFTR function and response to drugs (Dekkers et al., 2013, 2016a). The organoid response to a drug was calculated by subtracting the DMSO response at the same forskolin concentration.

Patient selection
A total of 24 patients (15 males and 9 females, median age 16.0 years) were included in this study. From these 24 patients, 15 patients had at least one S1251N mutation and were treated with CFTR modulators as part of a clinical trial aiming to compare different CFTR modulator treatments (NTR4586 and NTR4873). Thirteen of these 15 patients participated in both clinical trials and therefore received two different CFTR modifying treatments. The remaining 9 patients carried at least one rare CFTR mutation and were selected for off-label CFTR modulator treatments based on the organoid response and clinical necessity. A rare mutation was defined as a mutation with a prevalence of less than 1.0% in the Dutch CF population of which no data on clinical drug responsiveness was available in literature at the time of biopsy (Dutch Cystic Fibrosis Foundation, 2016). More information on the clinical characteristics of the selected patients is shown in Table 1. All patients (and/or their legal representatives) gave informed consent for rectal biopsies, generating and testing of their individual organoids as well as for (data collection on the effect of) clinical treatment.

Clinical endpoints
In vivo therapeutic effect in the patients with an S1251N mutation was measured by absolute change after 8 weeks of CFTR modulator treatment in comparison with pretreatment baseline data. From people with rare mutations receiving either ivacaftor or lumacaftor/ivacaftor was collected between 4-8 weeks after initiation of treatment. Forced expiratory volume in one second is a widely used readout to assess pulmonary function, and was expressed as percent predicted for body height, age and gender (ppFEV1). Sweat chloride concentration (SCC) measurements were assessed as this is currently the best established in vivo biomarker of CFTR function.

METHOD DETAILS

Forskolin-induced swelling of rectal organoids
Organoid swelling was measured in duplicate at multiple independent culture time points as indicated in Figure S1, with 4-8 different concentrations of forskolin as previously described (Dekkers et al., 2013, 2016a; Boj et al., 2017). The CFTR modulators (3 μM VX-770/ivacaftor (Selleck Chemicals LLC) or a combination of 10 μM genistein (Sigma) plus 50 μM curcumin (Sigma)) were directly added to the organoids with forskolin, except for VX-809/lumacaftor (3 μM, Selleck Chemicals LLC) that was pre-incubated for 24h. Organoids were fluorescently labeled and total area per well and time point was monitored by a Zeiss LSM800 confocal microscope. A Zen Image analysis software module (Zeiss) was used to quantify the organoid response (area under the curve measurements of relative size increase of organoids after 60 minutes forskolin stimulation, t = 0 min baseline of 100%).

Evaluation of clinical treatment
For all treatments both the patients and those who were involved in clinical data collection were blinded for the magnitude of the in vitro drug response of the patients’ organoids and vice versa. The ppFEV1 was measured according to ATS-ERS standards (American Thoracic Society, 1995; Beydon et al., 2007). The SCC was measured using the Macroduct® system and performed according to the most recent version of the standard operating procedure of the European Cystic Fibrosis Society-Clinical Trial Network.

QUANTIFICATION AND STATISTICAL ANALYSIS

The primary outcome of the study was the correlation (Pearson) between the in vitro organoid and in vivo effects (change in ppFEV1 and SCC) plus the predictive capacity of the organoid model, in patients that had a baseline ppFEV1 between 40 and 90 percent. When a change in ppFEV1 or SCC was missing, a patient was excluded from that part of the analysis. In a secondary analysis, we calculated the correlation and predictive capacity for patients that had a baseline ppFEV1 of < 40 or > 90 percent as well as for the total group of patients that was treated. Finally we used the wilcoxon signed rank test to examine the clinical response of patients with at least one rare CFTR mutation (non-F508del or S1251N) who had a response in their rectal organoids (AUC at 0.128 μM forskolin > 1000) to the CFTR modulating drug.

Receiver operating characteristic (ROC) curves were generated to evaluate the predictive capacity of organoid FIS for clinical responses. A Youden index was used to select the organoid cut-off point with the most optimal combination of sensitivity and specificity from the ROC-curves (Youden, 1950). A leave-one-out cross validation further validated our findings (Leeffang et al., 2008). As some patients were treated with two CFTR modifying treatments, we controlled for repeated measurements when calculating correlations and ROC-curves to evaluate a potential bias (Obuchowski, 1997; Lorenz, Datta and Harkema, 2011). Because of the limited number of patients, no further subgroup analysis were performed. Statistical analysis were performed using GraphPad Prism 7.02, IBM SPSS Statistics version 22 and R-studio version 0.99.441.
DATA AND SOFTWARE AVAILABILITY

All data is provided with the manuscript.

ADDITIONAL RESOURCES

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