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TITLE

Correction of CFTR function in intestinal organoids to guide treatment of Cystic Fibrosis

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RUNNING HEAD

CFTR function in intestinal organoids

CONTRIBUTORSHIP

Conceptualization: ASR, FV and KDB; Methodology: ASR, EF, JMB, AV, CVC, MF, FV, MP, MB, LD, MF recruiting of subjects and collection of rectal biopsies: MB, MP, FV, CVC, LD, IS, Belgian Organoid Project participants; Culturing the organoids and performing the FIS assay analysis: ASR,EF; Analysis of the results and figures preparation: ASR, FV; Writing – Original Draft: ASR, FV and KDB; Review & Editing: all; Supervision: FV and KDB.

ABSTRACT

Rationale

Given the vast number of *CFTR* mutations, biomarkers predicting benefit from CFTR modulator therapies are needed for subjects with cystic fibrosis (CF).

Objectives: To study CFTR function in organoids of subjects with common and rare *CFTR* mutations and evaluate correlations between CFTR function and clinical data.

Methods

Intestinal organoids were grown from rectal biopsies in a cohort of 97 subjects with CF. Residual CFTR function was measured by quantifying organoid swelling induced by forskolin and response to modulators by quantifying organoid swelling induced by CFTR correctors, potentiator and their combination. Organoid data were correlated with clinical data from literature.

Measurements and Main Results

Across 28 genotypes, residual CFTR function correlated tightly (r^2=0.87) with sweat chloride values. When studying the same genotypes, CFTR function rescue by CFTR modulators in organoids correlated tightly with mean improvement in lung function (r^2=0.90) and sweat chloride (r^2=0.95) reported in clinical trials. We identified candidate genotypes for modulator therapy, like E92K, Q237E, R334W and L159S. Based on organoid results, two subjects started modulator treatment: one homozygous for complex allele Q359K_T360K, and the second with mutation E60K. Both subjects had major clinical benefit.

Conclusions

Measurements of residual CFTR function and rescue of function by CFTR modulators in intestinal organoids correlate closely with clinical data. Our results for reference genotypes concur with previous results. CFTR function measured in organoids can be used to guide precision medicine in patients with CF, positioning organoids as a potential *in vitro* model to bring treatment to patients carrying rare *CFTR* mutations.

Introduction (2998/max 3000)

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (*CFTR)* gene coding for the CFTR protein that functions as an anion channel. More than 2000 *CFTR* mutations have been reported[1]. Only mutation F508del, occurring on 70 % of CF alleles, is frequent. In the European CF patient registry[2] just 5 mutations have a frequency above 1%. Many mutations are ultra-rare, occurring in just a few patients. In the CFTR2 project 412 mutations have been characterized so far[4].

Four CFTR modulators targeting the CFTR protein defect have been approved to treat patients: the correctors lumacaftor, tezacaftor and elexacaftor improve intracellular CFTR trafficking; the potentiator ivacaftor increases CFTR function. Corrector-rescued F508del-CFTR has impaired gating and requires a potentiator to optimize ion transport. The combination of one corrector plus potentiator results in modest clinical benefit for patients homozygous for F508del[5], while the combination of tezacaftor, elexacaftor and ivacaftor improved outcome in patients with one[6] or two F508del mutations[7]. Treatment with the potentiator ivacaftor brings major benefit for subjects with a class III mutation[8,9], and modest benefit to subjects with a selected list of residual function mutations[10]. For a review of this rapidly expanding area we refer to[11].

Most patients with rare *CFTR* mutations have at present no CFTR-directed treatment. Their small number is a hurdle for conclusive clinical trials. For these patients, the development of organoids as a biomarker of CFTR function and its rescue by CFTR modulators is a major breakthrough[12,13]. Although the culture conditions and techniques to grow organoids are complex, access to subject's rectal tissue via suction biopsies is easy. The procedure is painless and does not require local anesthesia. Organoids can be expanded over long time periods and biobanked[14]. In the context of CF, CFTR residual function and its rescue by CFTR modulators can be quantified by the forskolin induced swelling (FIS) assay[12,15]. Mean improvements seen in the different clinical trials correlate with CFTR rescue assessed

via the FIS assay in organoids of subjects with the trialed mutations[15]. In addition, organoid responses in subjects with ultra-rare mutations have predicted clinical benefit[15]. Results so far support that organoids can be used to guide personalized medicine[16].

We established a biobank of intestinal organoids of subjects with CF at the CF research lab of the University of Leuven (Belgium). Our aims were to assess the portability and robustness of the CF organoid model by confirming FIS assay results obtained in subjects with common mutations by others and to expand knowledge on organoid use in patients with rare *CFTR* mutations.

Materials and Methods

Subjects, rectal biopsies and mutations

This study was approved by the Ethics Review Board of the University Hospitals Leuven. All patients/parents gave written informed consent and/or assent. Rectal mucosa tissue was obtained by suction biopsy during a routine clinic visit. The biopsies were stored in ice cold phosphate buffer and kept on ice until crypts' isolation. In this cohort, no major adverse events were reported.

We recruited 97 subjects (Figure S1) with CF[17], with well characterized mutations (F508del, S1251N, R117H and G542X) and with rare *CFTR* mutations, including mutations not yet characterized in the CFTR2 project[4]. Clinical data retrieved from medical files are summarized in Table 1 and Table S1 and S2.

Isolation of crypts, culturing of organoids and forskolin induced swelling (FIS) assay

For detailed methods we refer to [15] and supplementary materials. Crypts were isolated from the rectal biopsies, subsequently mix with 50% Matrigel and plated on 24-well plates, medium has added after solidification of Matrigel as described before[12,18]. The growth medium was changed every other day. The organoids were split by mechanical disruption after every one week in culture. For 87% of the biopsies, organoids were successfully produced.

CFTR activity in the organoids was quantified using the FIS assay as described before[12]. In short, organoids (between 4^{th} and 20^{th} passage) were seeded in 96-well plates (Greiner) in 4 µL Matrigel drops containing 15–60 organoids covered with 50 µL of growth medium. The next day, calcein green (Invitrogen) was added for staining. Subsequently, to stimulate CFTR, 8 dilutions of forskolin from 0.008 to 5 µM were added and the organoids were immediately analyzed by confocal live-cell microscopy (LSM800, Zeiss, 5x objective). Every 10 min (from 0 to 60 min), the total organoid area (xy plane) was automatically quantified using Zen blue analysis software (Zeiss), and normalized to the area at t_0. To test rescue of

CFTR function by correctors, organoids were pre-incubated overnight with 3 μM lumacaftor (VX-809). Ivacaftor at a concentration of 3μM (VX-770, from Selleckchem) was added as a potentiator in combination with forskolin. Within each organoid experiment, every test condition was assessed in duplicate. Per organoid donor, 3 independent experiments were performed on different days. Values reported correspond to the average AUC (area under the curve) calculated from plots representing the mean percentage (%) of organoids swelling from t0 to t60 (60minutes) and standard error of the mean (SEM) of the 3 independent experiments (see supplementary data and Figure S2).

Residual CFTR function was determined from the organoid swelling after addition of forskolin alone. Rescue of CFTR function by CFTR modulator(s) was determined by the increase in AUC after stimulation with forskolin plus modulator(s) subtracting the increase after addition of forskolin alone.

Statistics

SPSS 23.0 or GraphPad were used for figures and statistical analysis. For correlations, Pearson correlation coefficients were calculated. A general linear model including subject, day of testing and their interaction was used for the variance component analysis of the response to modulators.

Results

The effect of lumacaftor and ivacaftor in organoids from subjects with mutations with known responses to CFTR modulators

FIS assays were performed in organoids from subjects with the following reference genotypes: F508del/S1251N (n=4, S1251N, the most common class III mutation in Belgium); F508del/R117H (n=2, R117H a class IV mutation); F508del/F508del (n=35, a class II mutation); G542X/G542X (n=1, a class I mutation). In F508del/S1251N and F508del/R117H organoids, maximal FIS was obtained after activation of CFTR by ivacaftor (Fig.1A), without additive effect from lumacaftor. F508del/R117H organoids showed a high residual CFTR function, as evidenced by the marked FIS without exposure to modulators. In F508del/F508del organoids, a modest FIS was seen after exposure to the combination of ivacaftor-lumacaftor. G542X/G542X organoids showed no response to CFTR modulators and no residual function. Fig.1B shows representative images of the swelling after 1 hour compared to baseline.

Correlating clinical trial results to organoid results

To isolate the drug effect from the residual CFTR function we subtracted the AUC with forskolin alone from the AUC with forskolin plus modulators. The mean improvements in FEV_1 and sweat chloride from published clinical trials [5,9,10,19–23] correlated closely with the mean FIS modulator responses seen in organoids from subjects with the same mutations as those in these trials (Fig.2B and 2C). The best correlations were found at forskolin concentration of 0.8 µM: r^2=0.90 between the responses in organoids and the mean changes in FEV_1 (Fig.2B), and r^2=0.95 between the responses in organoids and the mean changes in sweat chloride (Fig.2C). Correlations at forskolin concentrations of 0.32 µM were in the same range (Fig.S3).

Between- and within-subject differences in modulator responses for reference genotypes

FIS responses to the combination lumacaftor-ivacaftor in organoids from 35 F508del/F508del subjects ranged from very low, close to the absent response in the G542X homozygous subject, to as high as those seen with ivacaftor in F508del/S1251N organoids (Fig.3A). Subtraction of the forskolin effect had the most profound influence on the results from the R117H organoids (Fig.3B).

The within-subject repeatability of the FIS response to modulators is shown in Fig.3C: a high responder repeatedly is a high responder; a low responder repeatedly is a low responder, even within the group of F508del homozygous subjects ($p<0.001$). Variability in the response is mainly between subjects (68%), rather than between tests (24%) or within test (8%).

Residual CFTR function measured in organoids *in vitro* correlates with sweat chloride measured *in vivo*

In 74 subjects with 28 different genotypes we found an excellent semi-logarithmic correlation ($r^2=.87$) between the mean sweat chloride per genotype and the mean residual CFTR function for each genotype assessed in organoids (Fig.4A). For 5 subjects, sweat test results were not available.

Fig.4B upper panel displays residual CFTR function (FIS responses at 0.8 µM forskolin) in organoids from the 79 subjects with the 33 different genotypes studied (Table 1). The lower panel in Fig.4B represents the sweat chloride per genotype (with median and ranges reported in Table 1). Organoids with the highest residual function were from subjects with the lowest sweat chloride, the majority having values below the diagnostic threshold of 60 mmol/L (Fig.4B). Correlation was also observed with pancreatic status and age at diagnosis (Table S1). No correlation was found between the mean FEV1% predicted and the mean residual CFTR function (Fig.4C and Table S1).

Organoid swelling induced by CFTR modulators in subjects with rare *CFTR* mutations and F508del/minimal function mutations

FIS responses to CFTR modulators in organoids from 26 subjects with 23 different rare mutations and from 11 subjects heterozygous for F508del and a known minimal function mutation are shown in Fig.5. The responses in the 4 reference genotypes are displayed for comparison. The organoid swelling results induced by ivacaftor, lumacaftor and ivacaftor-lumacaftor combination (after subtracting the contribution of forskolin) are ranked from highest to lowest responders.

There was no correlation between residual function and response to CFTR modulators ($p=0.96$). The organoid response was higher than the average response to the combination lumacaftor-ivacaftor in F508del/F508del organoids for subjects with the E92K mutation and with the very rare mutations Q237E and Q359K_T360K (both absent in the CFTR2 database). Responses similar to those in F508del homozygous organoids were seen for L159S (also not explored in CFTR2) and for R334W.

For most genotypes, the swelling induced in organoids was higher with the combination ivacaftor-lumacaftor than with either drug alone. Only for mutations Q237E, D1152H, 3849+10kb(C<T) and R117C the response to ivacaftor equaled the response to the combination. E92K shows the particularity that CFTR function is mainly rescued by lumacaftor, with only a slight further increase on addition of ivacaftor (Fig.5).

Very high residual CFTR function but low additional responses to modulators were observed for mutation T5, classified as ‘of varying clinical consequence’ in the CFTR2 database.

Residual CFTR function was absent in all organoids with genotypes F508del/minimal function and at best low responses to lumacaftor plus ivacaftor were found.

Almost no response to modulators was observed in I1234V/W1282X organoids. For two mutations (g.3464_3471dupTCATTGCT_V1198M and K464E), not reported in CFTR1 nor in CFTR2, there was no swelling observed with the CFTR modulators tested.

Informing precision medicine: two examples

A 38year old pancreatic insufficient man with cystic fibrosis, homozygous for complex allele Q359K_T360K, received a donation for 1-month Orkambi™ treatment based on the organoid response (Fig.S4). Two weeks after starting treatment his FEV_1 rose from 49% to 65%, sweat conductivity decreased from 86 to 33 mmol/L and he gained 1.8 kg.

A high response to modulators was found in organoids from a subject with the E60K/I507del genotype (Figure S4 and Table S2), convincing the health authorities to approve treatment with Symkevi™. Six weeks after starting Symkevi™, sweat chloride improved from 73 to 36 mmol/L, FEV_1 from 32% to 47%, and CFQ-R_{resp} (a CF specific quality of life score to a maximum of 100 and a minimal clinically important difference of 4) from 24 to 78.

Discussion

We confirm and expand on previous findings by other labs in organoids from subjects with CF [12,13,15,24], showing that assessment of CFTR function in intestinal organoids is feasible, portable and repeatable, as these new results were obtained in a cohort of unique subjects, in a different lab by different researchers.

The magnitude of organoid responses seen in the reference genotypes is in line with those reported by Dekkers[15]. We also found a correlation between the mean clinical benefit from modulators in clinical trials and the mean rescue of CFTR function measured in organoids from subjects with the same mutations. However, the correlation with clinical trial data reported here is stronger than reported previously. By only taking into account the modulator response rather than the modulator plus forskolin response, we 'correct' for the high residual function seen in some organoids, mainly R117H. The closer correlation with clinical trial data in this paper (r^2=0.90 for changes in FEV_1) compared to Dekkers (r^2=0.76)[15] might also be related to larger numbers in the present study.

The within-subject repeatability of the FIS assay was acceptable, and much smaller than the between-subject variability. Organoid responses vary between subjects even within the same genotype. This mimics findings in the lumacaftor plus ivacaftor clinical trial in patients homozygous for F508del: the waterfall plot of improvement in FEV_1 shows large benefits in some subjects and hardly any in others[5] . Whether subjects whose response to modulators in organoids is higher than others with the same genotype can also be expected to have a better clinical response, is still to be investigated.

In subjects with 28 different genotypes, we showed an excellent (r^2=0.87) nonlinear correlation between sweat chloride and residual CFTR function assessed in organoids. Others also found correlations between *in vivo* and *in vitro* biomarkers of CFTR function, including sweat chloride concentration, CFTR mediated chloride transport by ICM in rectal biopsies and/or FIS on rectal organoids[13,25,26].

The logarithmic correlation is in line with McCague et al.[27], who showed a semilogarithmic correlation between sweat chloride of subjects with 226 different genotypes and the CFTR function in Fisher rat thyroid cells (FRT). Although the number of mutations assessed by McCague is much higher, the correlation (r^2=0.67) is not as tight as in our dataset (r^2=0.87). Indeed, organoids allow assessment of CFTR function in native tissue with the entire genetic background of the subject, compared to heterologous cell lines expressing only a mutant CFTR common to the subject, as is done in FRT cells. Furthermore, in FRT cells, the mutations are in the cDNA context without introns, jeopardizing the analysis of mutations that affect splicing and nonsense mRNA mediated decay (NMD)[28]. This favors organoids versus heterologous cell line models for the assessment of mutations and modulators on CFTR function. The results obtained for I1234V, E831X and R334W in organoids contradict observations in the FRT cells[29]. Organoid results are more in line with what is known about these mutations (see supplementary data). Discrepancies between findings in organoids and FRT cells have been reported before. No benefit of ivacaftor was seen in patients with a G970R mutation, behaving as an ivacaftor-responsive gating mutation in FRT cells[9]. Subsequently, no effect of ivacaftor was seen in organoids with the G970R mutation, and the mutation was shown to induce alternative splicing with very limited protein production[30]. This illustrates the superiority of using the patients' own tissue rather than heterologous expression of mutations in non-human cell lines.

The increase in CFTR function by modulators was also captured by means of ICM in native rectal tissue[31,32], with changes in CFTR mediated chloride transport correlated to changes in sweat chloride, but not in lung function. Residual CFTR function, measured by means of the FIS assay and of ICM[26], correlates with sweat chloride concentration. A correlation between FEV_1 and residual CFTR function on ICM was previously found[26]. The absence of correlation in our cohort could be explained by the larger age heterogeneity of our cohort or by fundamental differences in the physiology of the assay.

In organoids, both the correctors and the potentiators are added *in vitro*. ICM is performed on fresh biopsies resulting in faster results, the correctors have to be administered to the patients before executing the assay. The FIS assay does not allow comparison to wt-CFTR function, as pre-swelling of non-CF organoids results in little additional effect of CFTR activators or modulators. Organoid cultures can be stored in biobanks, allowing later re-testing when new drugs become available and easy exchanges between labs for remote testing or for research purposes.

We observed high levels of CFTR functional rescue in organoids of several subjects with rare mutations like E92K, Q237E and L159S (the last one not yet described in the CFTR2 database[4]. E92K is rescued by lumacaftor, concurring with results in heterologous cell systems[34,35]. FIS in E92K organoids was slightly higher with ivacaftor-lumacaftor than lumacaftor alone, suggesting impaired channel gating of rescued E92K. This common mutation in the Chuvash Russian population[36,37] would be a good candidate for combination modulator treatment.

We showed direct proof of clinical benefit in two subjects with high CFTR rescue in organoids, one homozygous for Q359K_T360K and the second with genotype E60K/I507del, the rescue being derived from the E60K allele. For the remaining genotypes, responses to modulators were in line with what was already known about the mutations from heterologous cell systems and *in silico* predictions (see supplementary data).

Our study has strengths by reporting on CFTR function in cultures derived from tissues of individual subjects in a large cohort with different genotypes. We showed excellent correlations between organoid responses to modulators and improvement in clinical trials. We also identified several rare mutations that may be amenable to treatment with the already approved modulators.

One weakness of our study is that we could correlate *in vitro* response to clinical improvement in only 2 subjects. The high cost of modulators and lack of approval for rare

mutations are still hurdles to the performance of therapeutic trials. For other rare mutations, we however provide convincing results of rescue in organoids backed up by knowledge of how these mutations disturb the normal CFTR structure or function.

Overall, our results confirm that the study of CFTR function and its rescue in rectal organoids opens a path to personalized therapies. This is especially relevant for patients with rare mutations unlikely to enter clinical trials, given the high number of rare mutations and the low number of subjects per mutation. Using organoids as biomarker to select responders to modulators opens a new horizon for these patients. We identify several new mutations that respond well to modulator therapies: some are ultra-rare (L159S, Q237E); others occur in hundreds of European patients like R334W and E92K.

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Legend of Figures

Figure 1 – Forskolin induced swelling (FIS) assay in the organoids from patients with reference genotypes ***A)*** *Organoids were incubated overnight with lumacaftor (VX-809 3μM). Stimulation with forskolin (increasing concentrations from 0.008 to 5μM) was done after 24 hours, either alone or in combination with ivacaftor (VX-770 3μM) during 1 hour. Each condition was tested in duplicate on three different days. Values plotted are the average and SEM of the AUC (area under the curve)* ***B)*** *Representative images of the organoids at baseline(t0) and after stimulation for one hour (t60) with forskolin without (DMSO) and with modulators. White scale bar in the first image corresponds to 200μm.*

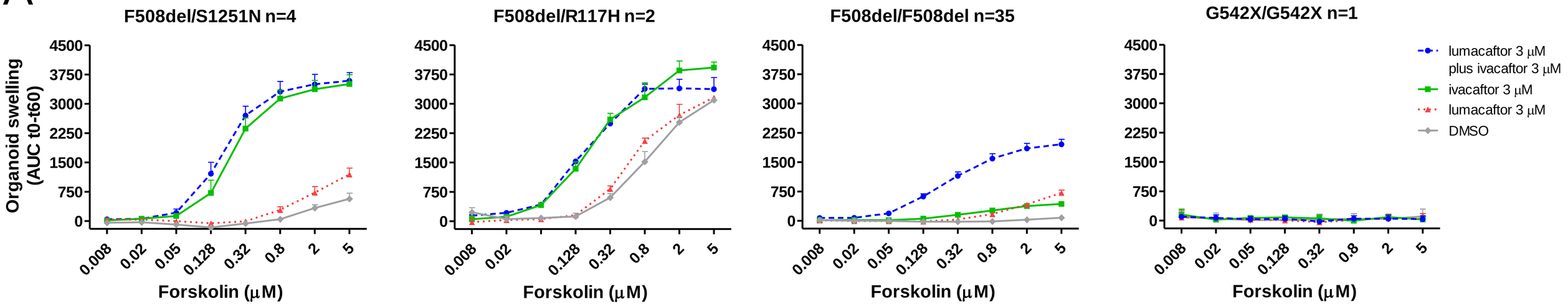
Figure 2 – ***Correlation between responses to modulators in vitro in the FIS assay and in vivo from published clinical trials. A)*** *Responses to lumacaftor and/or ivacaftor in organoids with the F508del/F508del, F508del/S1251N, F508del/R117H and F508del/minimal function (MF) were compared to the clinical responses (FEV_1 and sweat chloride) obtained in published clinical trials with patients having the same genotypes* ***B)*** *Correlation between the absolute change in FEV_1 in clinical trials and the organoid responses in our cohort at 0.8μM forskolin with the same modulators.* ***C)*** *Correlation between the absolute change in sweat chloride in clinical trials and the organoid responses in our cohort at 0.8μM forskolin with the same modulators.*

Figure 3 – ***Between- and within-subject variability of FIS in organoids of 42 patients with the reference genotypes. A)*** *Organoid swelling (AUC) with lumacaftor and ivacaftor at 0.8μM forskolin (without correction for the response to forskolin alone). (Mean and SEM for the 3 experiments per subject).* ***B)*** *Same as A after subtraction of the swelling (AUC) induced by forskolin alone.* ***C)*** *AUC at 0.8 μM forskolin for the combination of lumacaftor plus ivacaftor (corrected for the AUC at 0.8 μM forskolin without modulators) obtained in each of the 3 individual experiments on different days (Y-axis), according to the mean AUC of the 3 experiments (X-axis). Error bars represent SD of the two replicates within each experiment.*

Figure 4 – Correlation between ***residual CFTR function and sweat chloride. A****) Correlation between residual CFTR function measured in organoids after stimulation with 0.8μM forskolin alone and sweat chloride from subjects with the same genotype (Mean values per genotype). Sweat chloride was missing for 5 subjects with 5 different genotypes).* ***B)*** *The upper panel displays residual CFTR function (mean and standard error of the AUC at 0.8 μM forskolin without modulators) in organoids from 33 different genotypes (79 subjects) (Table 1). Genotypes are ordered from high to low residual CFTR function. The lower panel represents the sweat chloride for the same genotypes (individual value or mean and SD). Range of sweat chloride per genotype are reported in Table 1. The horizontal line represents the diagnostic cut-off for cystic fibrosis (60 mmol/L). Error bars represent the SEM of the 3 experiments (n=1) or the SEM of the different subjects (n>1). n=1 unless stated otherwise* ***C)*** *Correlation between residual CFTR function measured in organoids after stimulation with 0.8μM forskolin alone and FEV1% predicted from subjects with the same genotype (mean values per genotype). FEV1% predicted was missing for 25 subjects with 10 different genotypes.*

Figure 5 – ***Response to modulator(s****) at 0.8μM forskolin corrected for the residual function (response to forskolin alone) (red, green, blue), with the residual function (gray) stacked on top of the response to modulator(s). The four reference genotypes are placed on the left part of the graph (yellow), with genotypes ordered by the (mean) response to lumacaftor plus ivacaftor. The horizontal line corresponds to the mean response to lumacaftor plus ivacaftor in the organoids of the 35 F508del/F508del subjects. Error bars represent the SEM of the 3 experiments (n=1) or the SEM of the different subjects (n>1). Characteristic of the subjects are described in Table 1. n=1 unless stated otherwise*

A


F508del/S1251N n=4
F508del/R117H n=2
F508del/F508del n=35
G542X/G542X n=1
Organoid swelling (AUC t0-t60)
4500
3750
3000
2250
1500
750
0
0.008
0.02
0.05
0.128
0.32
0.8
2
5
Forskolin (μM)
lumacaftor 3 μM plus ivacaftor 3 μM
ivacaftor 3 μM
lumacaftor 3 μM
DMSO

B

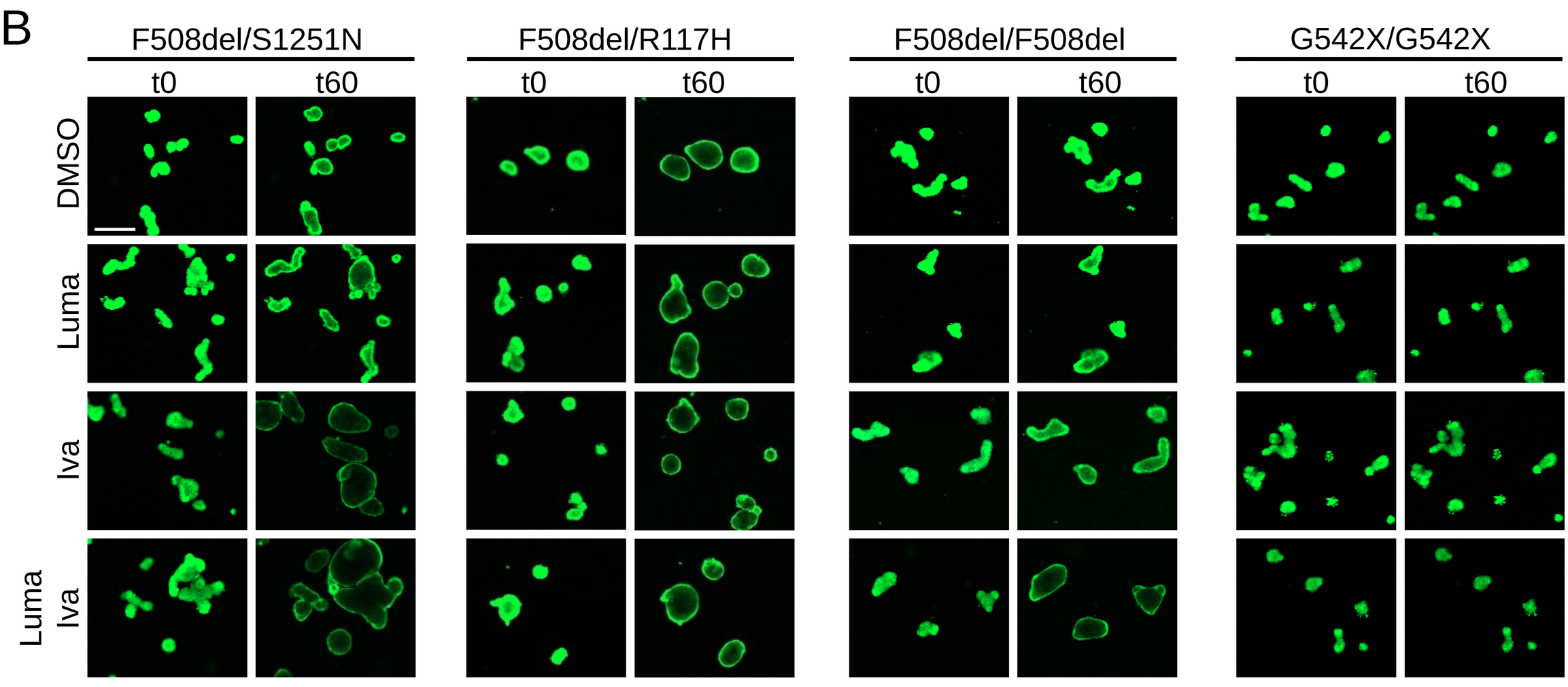

F508del/S1251N
F508del/R117H
F508del/F508del
G542X/G542X
t0
t60
DMSO
Luma
Iva
Luma Iva

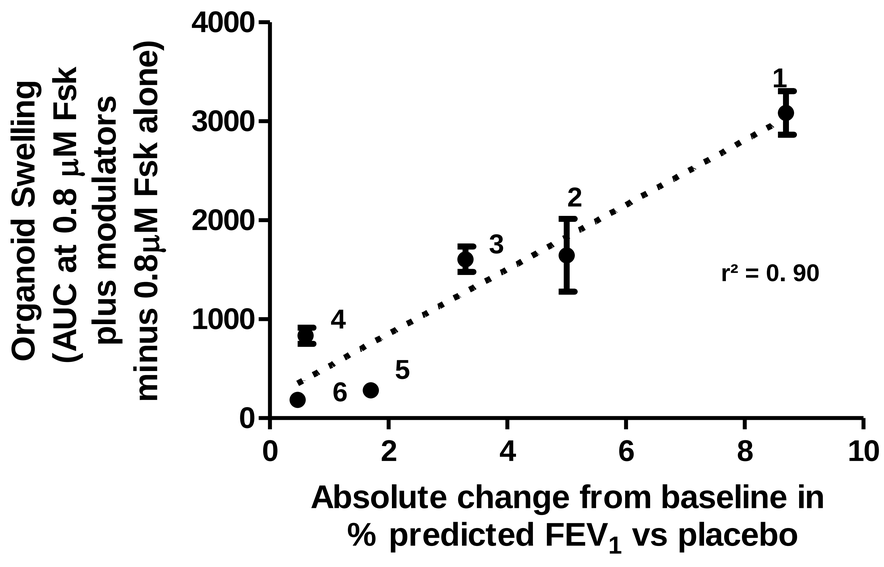
Fig.1

A

			Absolute change % predicted FEV_1 versus placebo	Absolute change from baseline in sweat chloride (mmol/L)	Organoid Swelling (AUC at **0.8 μM** Fsk plus modulators minus response 0.8 μM Fsk alone)	SEM	References
1	VX-770	S1251N n=4	8.7	− 54	3085	220	9
2	VX-770	R117H n=2	5.0	−24	1646	369	10
3	VX-809+ VX-770	F508del/F508del n=35	3.3	-21	1607	126	5[a], 23[b]
4	VX-809+ VX-770	F508del/MF n=10	0.6	-11	833	82	20
5	VX-770	F508del/F508del n=35	1.7	-3	282	40	21
6	VX-809	F508del/F508del n=35	0.5	−8	172	29	22

[a] reference for FEV_1 value; [b] reference for sweat chloride value

B



C

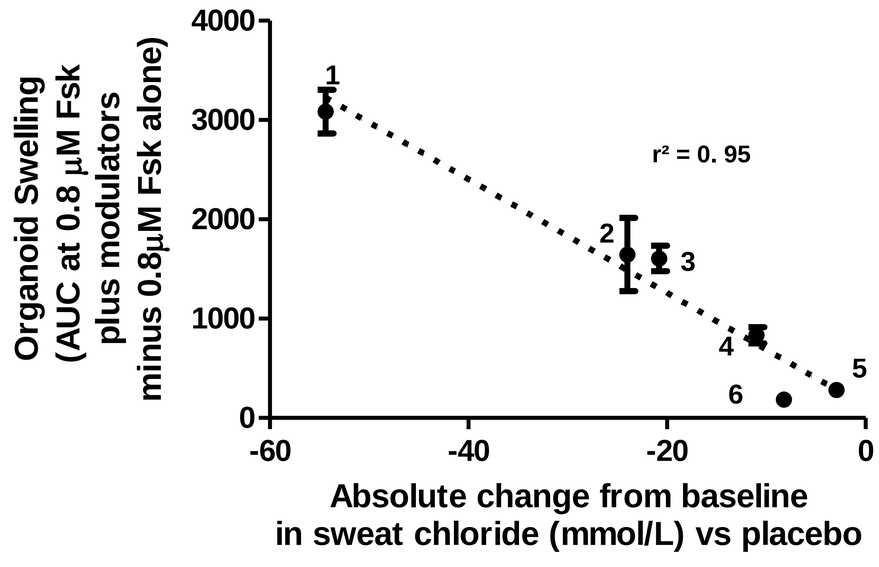
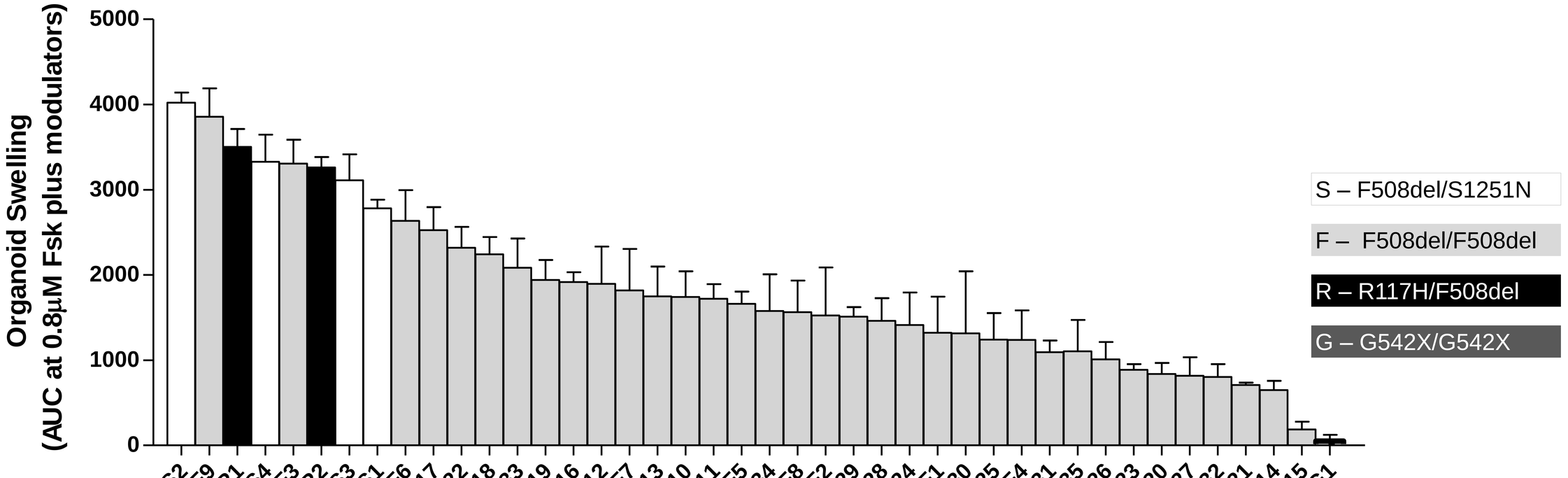


Fig.2

A

Response to lumacaftor and ivacaftor



B

Response to lumacaftor and ivacaftor substracting effect of forskolin

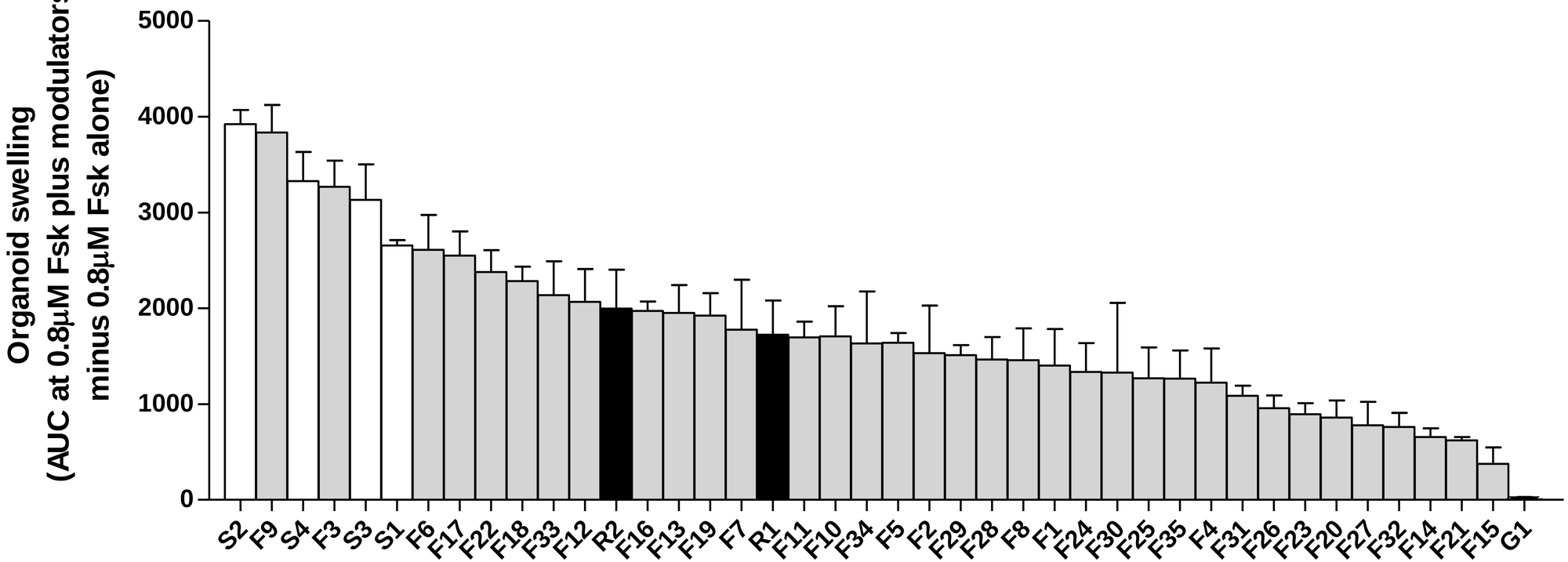


Fig.3A and B

Responses to lumacaftor plus ivacaftor and forskolin (0.8μM) minus forskolin (0.8μM) alone (reference genotypes)

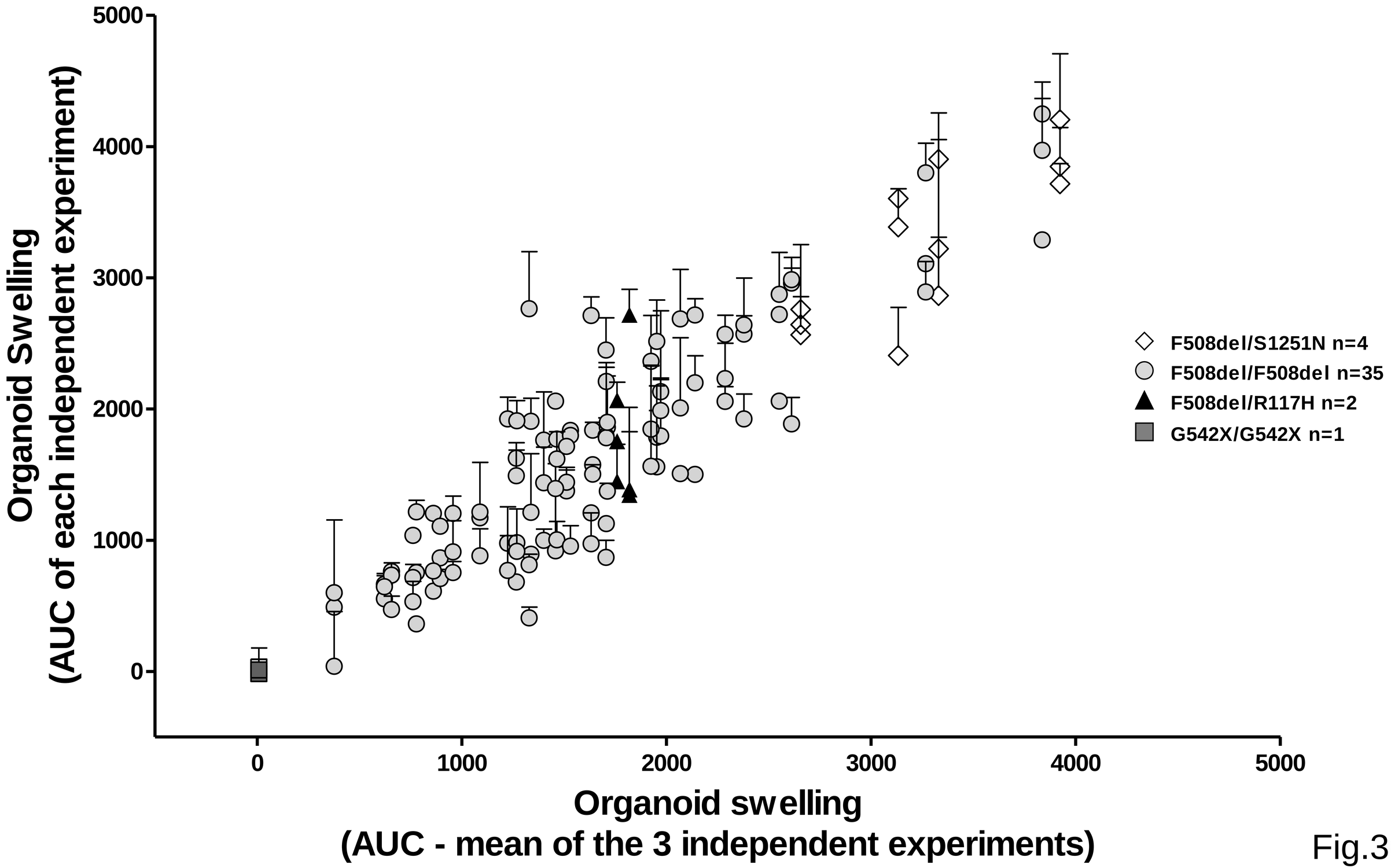


Fig.3C

A

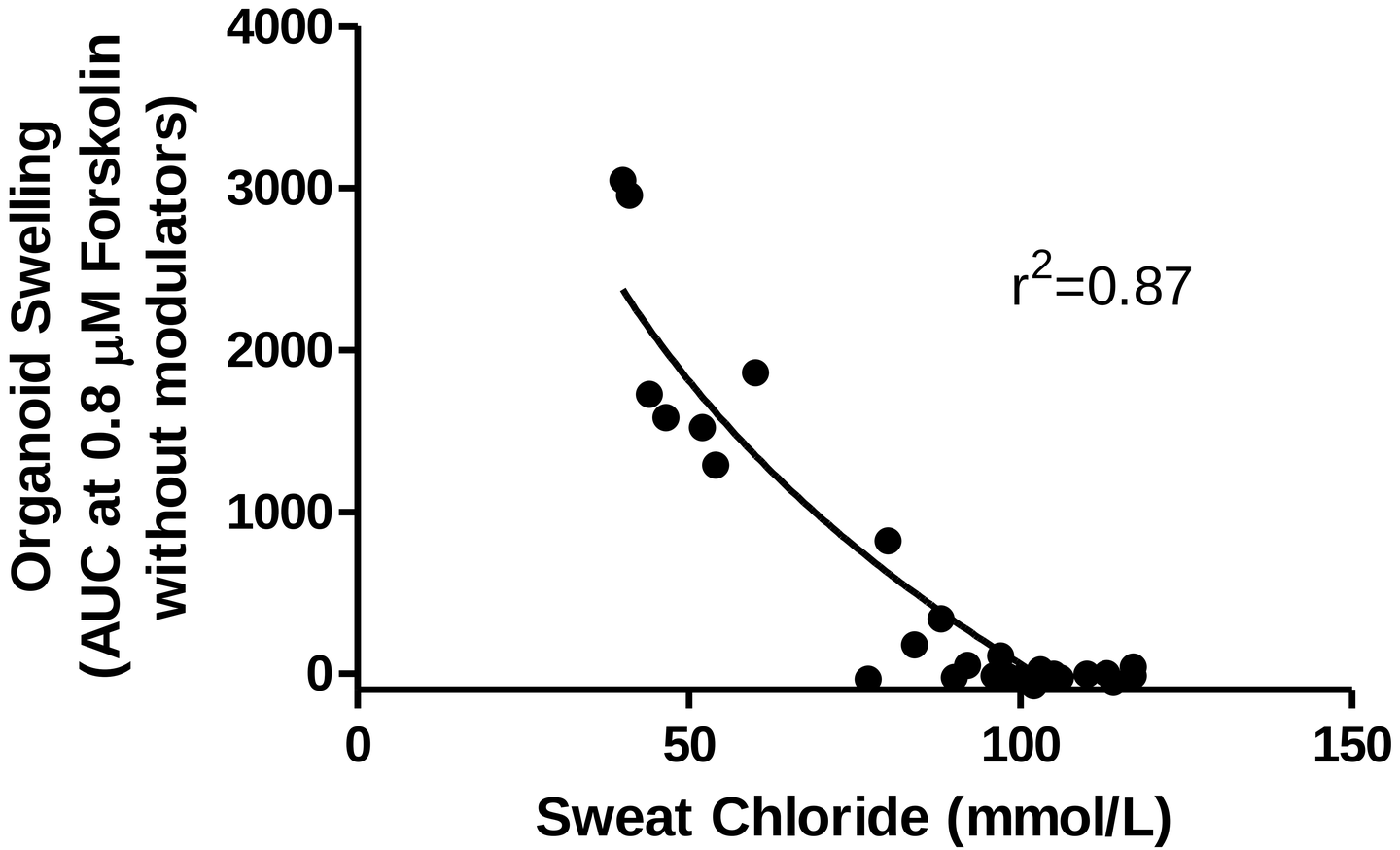

Organoid Swelling
(AUC at 0.8 µM Forskolin
without modulators)
4000
3000
2000
1000
0
0
50
100
150
Sweat Chloride (mmol/L)
r2=0.87

Fig.4A

B

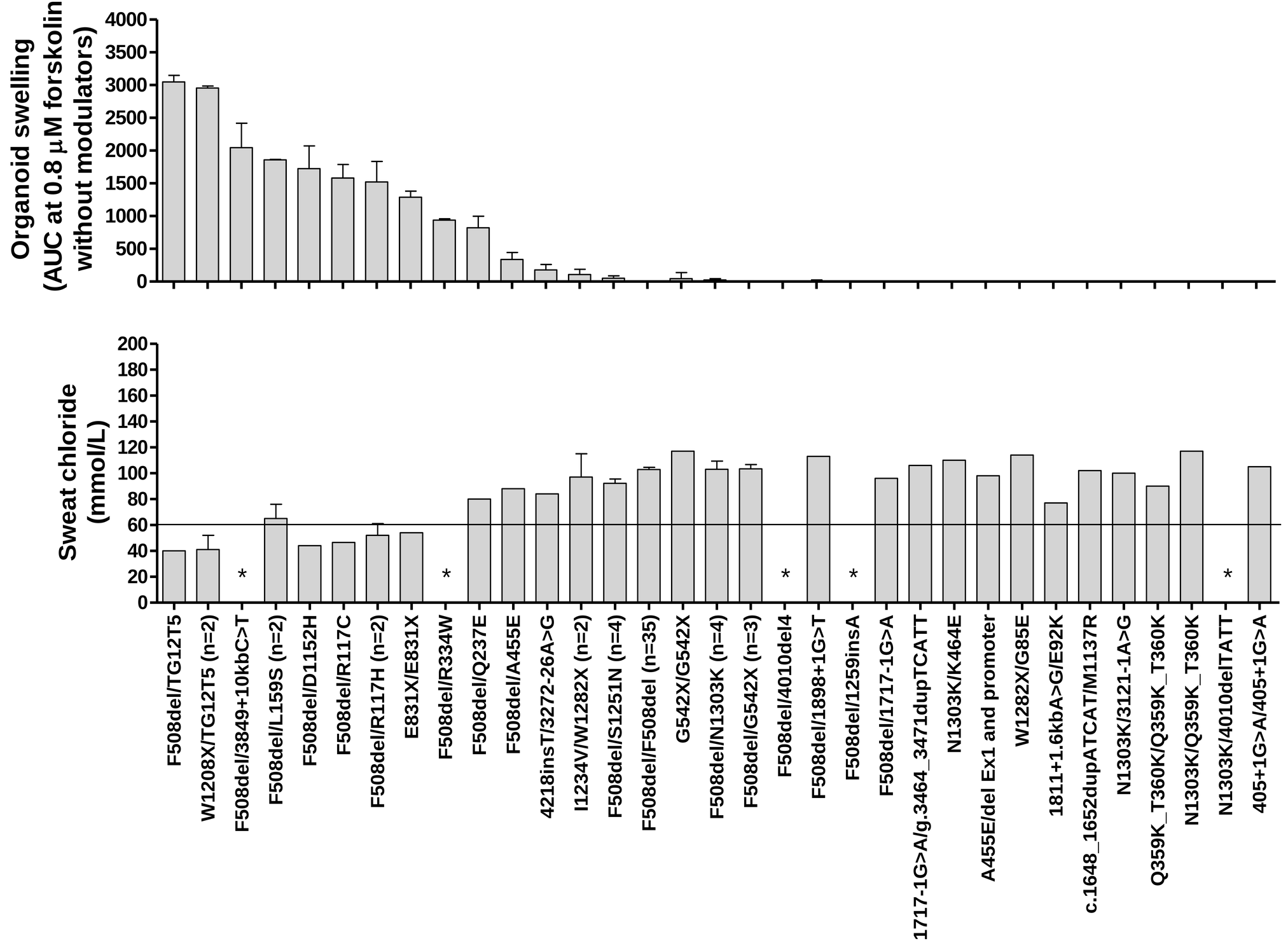

Organoid swelling (AUC at 0.8 µM forskolin without modulators)
4000
3500
3000
2500
2000
1500
1000
500
0
Sweat chloride (mmol/L)
200
180
160
140
120
100
80
60
40
20
0
*
*
*
*
F508del/TG12T5
W1208X/TG12T5 (n=2)
F508del/3849+10kbC>T
F508del/L159S (n=2)
F508del/D1152H
F508del/R117C
F508del/R117H (n=2)
E831X/E831X
F508del/R334W
F508del/Q237E
F508del/A455E
4218insT/3272-26A>G
I1234V/W1282X (n=2)
F508del/S1251N (n=4)
F508del/F508del (n=35)
G542X/G542X
F508del/N1303K (n=4)
F508del/G542X (n=3)
F508del/4010del4
F508del/1898+1G>T
F508del/1259insA
F508del/1717-1G>A
1717-1G>A/g.3464_3471dupTCATT
N1303K/K464E
A455E/del Ex1 and promoter
W1282X/G85E
1811+1.6kbA>G/E92K
c.1648_1652dupATCAT/M1137R
N1303K/3121-1A>G
Q359K_T360K/Q359K_T360K
N1303K/Q359K_T360K
N1303K/4010delTATT
405+1G>A/405+1G>A

Fig.4B

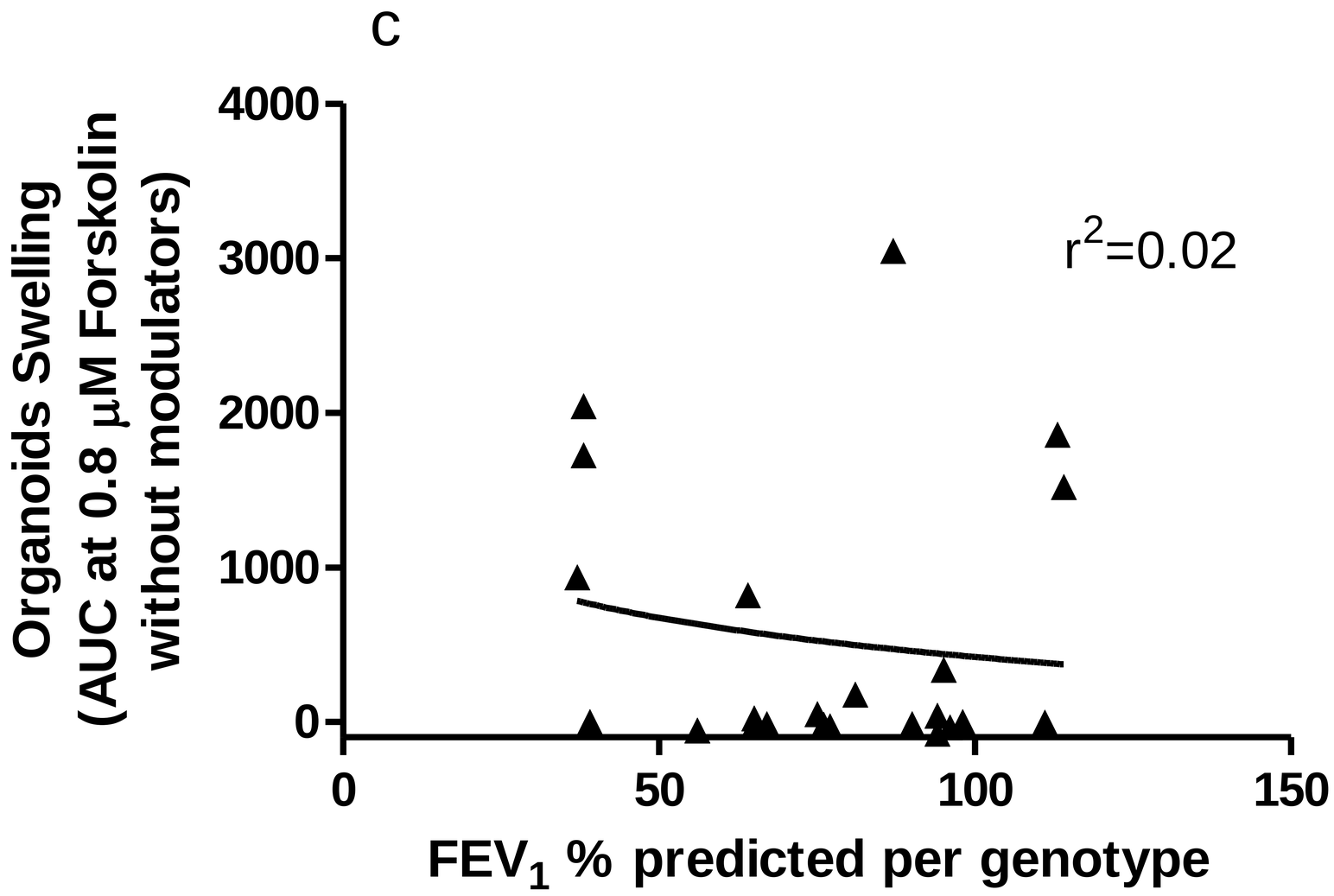

C
Organoids Swelling
(AUC at 0.8 µM Forskolin
without modulators)
4000
3000
2000
1000
0
0
50
100
150
FEV1 % predicted per genotype
r2=0.02

Fig.4C

response to forskolin alone (stacked)

response to lumacaftor and forskolin minus response to forskolin alone

response to ivacaftor and forskolin minus response to forskolin alone

response to lumacaftor plus ivacaftor and forskolin minus response to forskolin alone

Organoid Swelling (AUC at 0.8µM forskolin)

5000
4000
3000
2000
1000
0

F508del/S1251N (n=4)
F508del/R117H (n=2)
F508del/F508del (n=35)
G542X/G542X
1811-1.6KbA>G/E92K
F508del/Q237E
[Q359K_T360K]/[Q359K_T360K]
F508del/L159S (n=2)
F508del/R334W
A455E/delEx1
F508del/A455E
F508del/D1152H
W1282X/T5 (n=2)
F508del/3849+10kb(C>T)
F508del/1259insA
c.1648_1652dupATCAT/M1137R
F508del/1717-1G>A
F508del/R117C
F508del/1898+1G>C
F508del/G524X (n=3)
F508del/N1303K (n=4)
F508del/TG12T5
F508del/4010del4
N1303K/[Q359K_T360K]
4218INST/3272-26A>G
W1282X/I1234V (n=2)
E831X/E831X
N1303K/4010delTATT
N1303K/K464E
1717-1G>A/[g.3464_3471dupTCATTGCT_V1198M]
N1303K/3121-1G>A
W1282X/G85E
405+1G>A/405+1G>A

Fig.5

TABLES

***Table* 1–** Baseline characteristics of subjects from whom the rectal organoids are included in the study with lumacaftor and ivacaftor

Genotype	n	Gender	age (yrs) Median (range)	Age at diagnosis (yrs) Median (range)	Sweat [Cl-] (mmol/L) Median (range)	PS/PI	FEV_1 % predicted Median (range)	CFTR1 info	CFTR2 info; n reported
- reference genotypes									
F508del/F508del	35	M(22) F(13)	9 [1-46]	0.2[a] [0-7.5]	102[b] [86.5-119]	PI	76[c] [28-137]	yes	CF-causing; n=33983
F508del/S1251N	4	M(2) F(2)	21.5[12-37]	0.7 [0.1-0.8]	94[83-98]	PI(3)PS(1)	75 [52-107]	yes	CF-causing; n=92
F508del/R117H	2	M	19-27	11.5-19.7	43-61	PS	117-111	yes	variable consequences, n=1309
G542X/G542X	1	F	41	1.6	117	PI	94	yes	CF-causing; n=121
- F508del heterozygous									
F508del/MF (F508del/N1303K n=4, F508del/G542X n=3, F508del/4010del4 n=1, F508del/1898+1G>C n=1, F508del/1259insA n=1,F508del/1717-1G>A n=1)	11	M(3) F(8)	11.5 [5-43]	0.3[d][0-10.1]	101[d][90-121]	PI	82[e][30-102]	yes	all CF-causing, F508del/N1303K n=1240, F508del/G542X n=2112, F508del/4010del4 n=5, F508del/1898+1G>C n=292, F508del/1259insA n=7 and F508del/1717-1G>A n=800)
F508del/R117C	1	M	5	3.5	46.5	PS	ND	yes	CF-causing, n=91
F508del/L159S	2	F	18-20	4.7-6.6	76-54	PS	113	yes	L159S not reported
F508del/Q237E	1	M	48	39.1	80	PS	64	yes	Q237E not reported
F508del/R334W	1	M	59	19.4	ND	PI	37	yes	CF-causing, n=231
F508del/A455E	1	F	10	0.2	88	PI	95	yes	CF-causing, n=355
F508del/D1152H	1	M	57	44.6	44	PS	38	yes	variable consequences, n=358
F508del/TG12T5	1	M	14	2.9	40	PS	87	yes	variable consequences, n=95
F508del/3849+10kbC>T	1	F	31	8.2	ND	PS	38	yes	CF-causing, n=651
- non F508del									
Q359K_T360K/Q359K_T360K	1	F	40	ND	90	ND	ND	yes	not reported
A455E/del Ex1 and promoter	1	M	52	27.9	98	PS	65	yes	del ex1 and promotor not reported, A445E CF-causing n=499
E831X/E831X	1	F	2	0.3	54	PS	ND	yes	CF-causing, n=2
W1282X/G85E	1	M	34	0.4	114	PI	56	yes	CF-causing, n=10
W1282X/I1234V	2	F(1) M(1)	26-31	ND	79-115	ND	ND	yes	CF-causing, n=9
W1282X/TG12T5	2	F(1) M(1)	10-13	ND	30-52	ND	ND	yes	combination not reported, W1282X CF-causing n=1556, TG12T5 variable
N1303K/Q359K_T360K	1	F	18	ND	117	ND	ND	yes	combination not reported, N1303K CF-causing n=2147, Q359K_T360K not reported
N1303K/K464E	1	F	13	0.3	110	PI	111	K464E not reported	combination not reported, N1303K CF-causing n=2147, K464E not reported
N1303K/3121-1G>A	1	M	13	ND	100	PI	ND	yes	CF-causing, n=1
N1303K/4010del4	1	F	23	ND	ND	ND	ND	yes	CF-causing, n=1
405+1G>A/405+1G>A	1	M	27	ND	105	ND	ND	yes	CF-causing, n=2
c.1648_1652dupATCAT/M1137R	1	M	11	0.2	102	PS	94	c.1648_1652dupATCAT not reported	both not reported
1717-1G>A/ g.3464_3471dupTCATTGCT and V1198M	1	F	16	0.9	106	PI	77	g.3464_3471dupTCATTGCT and V1198M not reported	g.3464_3471dupTCATTGCT and V1198M not reported, 1717-1G>A CF-causing n=1202
1811+1.6kbA>G/E92K	1	F	6	0.4	77	PI	96	yes	combination not found, both CF-causing, E92K n=42, 1811+1.6kbA>G n=22
4218insT/3272-26A>G	1	M	40	0.1	84	PI	81	yes	CF causing, n=1

Note: a - only 33, b-only 32, c-only 24, d-only 10 and e- only 8

Supplementary data

Supplementary methods

Calculation of AUC for the FIS assay (see supplementary Figure S2)

Two wells are used for each condition. Images of each well are taken at 10 minutes interval during the FIS assay, and typically contain 15 to 60 organoids. The total organoid area is calculated from each picture, and normalized to the area at t0 (considering area at t0 as 100%). This normalized area is plotted vs time, to calculate the AUC (area under the curve) for each of the wells.

For each experiment, the mean of the AUCs of the 2 wells is calculated for each of the forskolin concentrations and modulators used. The experiment is repeated on three different days, and the final AUC reported is the mean (and SEM) of the AUCs from the three experiments. The final graph summarizes the changes in AUC for each forskolin concentration and modulator (combination).

Supplementary discussion

Supplementary information about rare mutations analyzed in this study

The results on CFTR rescue in the organoids of CF patients with different rare mutations observed in this study correlates well with the position, structural and functional information previously reported about these mutations, as described below.

Mutation E92K [1], located in the first transmembrane (TM1) part of membrane spanning domain 1 (MSD) is thought to abrogate a salt bridge needed for correct protein folding [2]. CFTR function was rescued by lumacaftor in the organoids, suggesting E92K is a class II mutation, concurring with results in heterologous cells systems [2,3] showed to be a

processing/trafficking mutant as E60K [4]. E92K was also rescued by correctors GLP2222 [5], FLD304 and FLD160 [6] supporting the folding defect.

The L159S mutation has been reported in the CFTR1 database in a pancreatic sufficient patient diagnosed at 3 months of age. Both patients tested in this study are also pancreatic sufficient, and one has a sweat chloride in the intermediate range in line with a high residual function observed in the FIS assay. This mutation lies on the first intracellular loop of MSD1 within 5A of a putative docking site for corrector molecules [7]. The Q237E position, also in MSD1 (TM4), being in a transmembrane domain, may be involved in pore formation. Thus, there may be alterations in the gating properties of the CFTR channel when this position is mutated. The FIS assay of this mutant protein showed some residual function but mainly high rescue of CFTR function with ivacaftor, which may reflect the rescue of the putative gating defect.

We found residual CFTR function and rescue of function by ivacaftor and lumacaftor- ivacaftor in F508del/R334W organoids. The R334W mutation, located in TM6 of MSD1, reduced single-channel conductance by ~60% by impeding ion-ion interactions within the CFTR pore [8], thus suggesting disturbed gating or conductance. This is in agreement with the FIS results where most of the CFTR rescue came from ivacaftor. In contrast, in FRT cells, this mutation is reported as associated with normal CFTR expression, but very low function and no rescue of function by ivacaftor [9]. Hence assessment of benefit from modulators seems needed in subjects.

We noted moderate responses to ivacaftor in organoids with mutations A455E, D1152H, 3849+10Kb C>T and R117C. Also when expressed in FRT cells, these mutations have >10% improvement in CFTR function [9], findings that led to FDA approval for ivacaftor treatment for these mutations [10].

We observed considerable residual function for mutation E831X, however no rescue with the modulators, in accordance with E831X being a splicing mutation that induces exon 14b skipping in 76% of transcripts, a premature stop codon in 16% and lack of one amino acid in

8% [11]. The latter transcripts result in a functional protein, that may be responsible for this residual function observed in organoids homozygous for this mutation.

We found almost no response to modulators in I1234V/W1282X organoids although in CFTR2, I1234V is annotated as having 107% expression and 40% of wild type function in FRT cells. In fact, other studies already showed that this is not a missense mutation but a splicing mutation and the resulting truncated protein lacking 6 amino acid from the N-terminal portion of second nucleotide binding domain (NBD2) has very low function [12,13].

Organoid results for mutations R334W and E831X contradict findings in FRT cells. Discrepancies between findings in organoids and FRT cells have been reported before. Based on data in FRT cells, mutation G970R was included in the clinical trial assessing the effect of ivacaftor in patients with non G551D gating mutations. However, a benefit was seen in all subjects except those with the G970R mutation [14]. Subsequently rescue of CFTR function by ivacaftor was proven absent in organoids of patients with the G970R mutation and the mutation was proven to induce alternative splicing [15]. This demonstrates the superiority of using the patients' own tissue rather than heterologous expression of mutations in non-human cell lines.

We highlight 2 non-characterized non-responding/swelling mutations, 1648_1652dupATCAT and K464E. The duplication 1648_1652dupATCAT induces alterations in the reading frame leading to a premature stop codon and resulting in no production of normal CFTR protein. In K464 (lysine) localized in the walker A region of the first nucleotide binding domain is completely conserved in CFTR sequences across different species [16]. This amino acid is thought crucial for the ATP binding [17].

Lumacaftor and ivacaftor was the treatment used for all the analysis in the paper except for the patients that were treated clinically, for which the FIS assays were done posteriorly and at that time were already using tezacaftor (also 3μM) and ivacaftor. The number of assays done

with teza/iva combination was very reduce, not allowing similar correlation as the ones done for the luma/iva.

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SUPPLEMENTARY TABLE

Supplementary Table S1 – *Comparison of clinical features between subjects with and without residual function when stimulated with 0.8 μM of forskolin.*

	Residual function	No residual function	p value
Genotypes (patients)	13 (17)	20 (62)	
AUC at 0.8μM forsolin alone	1416 ± 262.5 (n=17)	-11.73 ± 6.560 (n=62)	0.0002[a]
age (yrs)	25.29 ± 4.185 (n=17)	17.92 ± 1.572 (n=62)	0.1[b]
Age at diagnosis (yrs)	12.37 ± 4.066 (n=13)	1.519 ± 0.5689 (n=54)	0.0003[c]
Sweat [Cl-] (mmol/L)	63.10 ± 5.945 (n=15)	102.5 ± 1.298 (n=57)	< 0.0001[b]
Pancreatic sufficiency (n [%])	10 [59%][d]	3 [5%][e]	< 0.0001[f]
FEV1 % predicted	81.27 ± 9.723 (n=11)	77.74 ± 3.805 (n=43)	0.7[b]

Data are shown as mean ± SEM and/or percentage. [a]Unpaired t test with Welch's correction. [b]Unpaired t test. [c]Mann–Whitney test.[d]For 4 [24%] subjects with residual function the value of pancreatic status was not available. [e]For 4 [6%] subjects without residual function the value of pancreatic status was not available. [f]chi-square test

Supplementary Table S2 – Baseline characteristics of subjects included in supplementary Fig.S4, whose organoids were tested with tezacaftor and lumacaftor.

Genotype	n	Sex	age (yrs) Median (range)	Sweat [Cl-] (mmol/L) Median (range)	PS/PI	FEV_1 % predicted Median (range)	CFTR1 info	CFTR2 info; n reported
F508del/F508del	15	M(7) F(8)	18 [2-44]	107 [83-127]	PI	71.2 [36-124]	yes	CF-causing; n=33983
F508del/S1251N	1	M	55	91	PI	45	yes	CF-causing; n=92
I507del/N1303K	1	M	22	102	PI	50-60	yes	CF-causing; n=14
E60K/I507del[a]	1	F	25	73	PI	32	yes	CF-causing; n=1

a-This 25-year-old pancreatic sufficient woman was diagnosed with CF at the age of 5.4 years due to chronic respiratory symptoms, bronchiectasis and Pseudomonas aeruginosa colonization. Despite intensive symptomatic treatment, she had recurrent hemoptysis during many years and a steep downhill course in FEV1 from 80% in 2008 to 32% in 2017.