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Extent of rescue of F508del-CFTR function by VX-809 and VX-770 in human nasal epithelial cells correlates with SNP rs7512462 in *SLC26A9* gene in F508del/F508del Cystic Fibrosis patients

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ABSTRACT

Background: We analyzed the CFTR response to VX-809/VX-770 drugs in conditionally reprogrammed cells (CRC) of human nasal epithelium (HNE) from F508del/F508del patients based on SNP rs7512462 in the Solute Carrier Family 26, Member 9 (*SLC26A9*; MIM: 608481) gene.
Methods: The $I_{sc\text{-}eq}$ measurements of primary nasal epithelial cells from F508del/F508del patients (n = 12) for CFTR function were performed in micro Ussing chambers and compared with non-CF controls (n = 2). Data were analyzed according to the rs7512462 genotype which were determined by real-time PCR.
Results: The CRC-HNE cells from F508del/F508del patients evidenced high variability in the basal levels of CFTR function. Also, the rs7512462*C allele showed an increased basal CFTR function and higher responses to VX-809 + VX-770. The rs7512462*CC + CT genotypes together evidenced CFTR function levels of 14.89% relatively to wt/wt (rs7512462*CT alone-15.29%) i.e., almost double of rs7512462*TT (7.13%). Furthermore, sweat [Cl^-] and body mass index of patients also evidenced an association with the rs7512462 genotype.
Conclusion: The CFTR function can be performed in F508del/F508del patient-derived CRC-HNEs and its function and responses to VX-809 + VX-770 combination as well as clinical data, are all associated with the rs7512462 variant, which partially sheds light on the generally inter-individual phenotypic variability and in personalized responses to CFTR modulator drugs.

1. Introduction

Cystic fibrosis (CF; MIM: 219700) is caused by pathogenic variants in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*; MIM: 602421) gene [1–3] encoding a chloride (Cl^-) and bicarbonate ($HCO3^-$) channel that functions at the apical membrane of epithelial cells. CFTR is essential for the ionic balance and homeostasis of the epithelial fluid covering epithelial tissues, namely the airway surface liquid (ASL) in the airways, and negatively regulates the sodium (Na^+) epithelial channel (ENaC). In the absence of functional CFTR, ENaC is hyperactive, leading to increased Na^+ and water uptake by the epithelium, with consequent ASL dehydration [2,4–8]. However, besides the airways, defective CFTR function results in complications in several organs, including the pancreas, intestine, liver, sweat gland and male reproductive system [9–14].

In CF, ~2030 variants are described in the *CFTR* gene, of which ~300 have been annotated as CF-causing and are classified into 7 functional groups (I-VII) for a better strategy towards precision medicine aimed at treating the underlying distinct molecular/cellular defects that cause CF disease. Nevertheless, F508del (p.Phe508del, c.1521_1523delCTT, c.1521_1523del or 1653delCTT) is the most frequent mutations, occurring in ~85% of all CF patients worldwide at

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least in 1 allele [7,8,14–18].

Several drugs are used for the treatment of individual CF symptoms, and these have significantly increased life quality and expectancy of individuals with CF [19]. However, CFTR-modulating drugs that correct the CFTR basic defect, are already in the clinic [8,15]. Among these drugs which were already approved in the USA and in some European countries, are Ivacaftor®/VX-770 (Kalydeco®) for some class III/IV mutations [20,21] and the combinations of Lumacaftor®/VX-809 with Ivacaftor®/VX-770 (Orkambi®) for F508del homozygous individuals [22–24]. More recently, Tezacaftor®/VX-661 in combination with Ivacaftor® (Symdeko®) was approved in the USA for CF patients who are F508del-homozygous and found to be efficacious in CF patients who are heterozygous for F508del and a CFTR residual-function mutation [25,26].

It is known for long that individuals with the same *CFTR* genotype have distinct CF phenotypes [22–27], including different manifestations in the affected organs. Environmental factors, CF-modifying genes and stochastic factors determine the way CF develops [27–30]. Furthermore, the treatment of CF individuals bearing the same *CFTR* genotype with CFTR modulator drugs have also demonstrated a high inter-individual variability in terms of clinical benefit [20–26].

In this context, multiple genetic variants have been studied to evaluate their potential influence on response variability [27–29], and recently one SNP (rs7512462), in the Solute Carrier Family 26 Member 9 (*SLC26A9*; MIM: 608481) gene, was shown to modulate both the patient's response and the functional CFTR response in primary human bronchial epithelial (HBE) cells to CFTR modulator drugs, VX-770 and VX-809 [30]. A previous study had shown that cells expressing F508del-CFTR evidence reduced plasma membrane expression of SLC26A9 when compared to those expressing wt-CFTR [31].

Against this background, the current study aimed to analyze CFTR function and the response to VX-809/VX-770 combination drugs in conditionally reprogrammed cells (CRC) of human nasal epithelial (HNE) collected from F508del/F508del patients and establish a correlation between the magnitude of the response with the allelic variants at the SNP rs7512462 in the *SLC26A9* gene.

2. Material and methods

2.1. Ethics

The participants of this study signed the Informed Consent Form under approval of the Ethics Committee from the University of Campinas (#91.041.317). The procedures performed are in accordance with the Helsinki Declaration.

2.2. Collection of human primary nasal epithelial cells (HNE) and culture of conditional reprograming cells of human nasal epithelial (CRC-HNE)

HNE cells were collected from 12 F508del/F508del patients and 2 controls (wt/wt), that were not on use of the CFTR's modulators (VX-809 and VX-770), with an interdental brush after nasal washing with 0.9% (w/v) saline solution, as described [32]. Cells were collected into DMEM-F12 culture medium (Sigma-Aldrich/D9785, Merck KGaA, Darmstadt, Germany) supplemented with 1% L-Glutamine (Sigma-Aldrich/G7513), 1% penicillin and streptomycin (Sigma-Aldrich/P4333), 10% new born calf serum (Sigma-Aldrich/N4637), 0.4% Amphotericin B (Sigma-Aldrich/A2942), 0.16 μg/mL Tobramycin (Sigma-Aldrich/T4014) and Vancomycin 0.1 μg/mL (Sigma-Aldrich/V2002). The cells were then grown to 70–90% confluency, as previously described [33]. After trypsinization CRC-HNE cells were counted and transferred to transmembrane filters (Corning® Costar® Snapwell cell culture inserts – Sigma-Aldrich/CLS3801) and cultured in air-liquid interface (ALI) as described [34] for ~15–30 days, until the transepithelial resistances (TEER) values were higher than 1000 Ω (Milicell® ERS-2). Then polarized HNE cultures were incubated with 3 μM VX-809 (Selleckchem/S1565, Houston, TX, USA) or 0.05% (v/v) DMSO (Sigma-Aldrich/D2650) as vehicle control for 48 h.

2.3. Measurement of CFTR activity in CRC-HNE cells by micro Ussing chamber

CRC-HNE cell monolayers were mounted on micro-Ussing chambers and analyzed under open-circuit conditions at 37 °C as previously described [34]. Values for the transepithelial voltage (V_{te}) were referenced to the basal surface of the epithelium. The transepithelial resistance (R_{te}) was determined by applying 1 s current pulses of 0.5 μA (5 s – period) and the cAMP–stimulated CFTR equivalent short-circuit currents ($I_{sc\text{-}eq}$) were calculated according to Ohm's law ($I_{eq\text{-}sc} = V_{te}/R_{te}$) with appropriate correction for fluid resistance, as described [35]. Ringer's buffer solution ((mM): 145 NaCl, 0.4 K_2HPO_4, 1.6 $K_2HPO_4.3H_2O$, 5 D-glucose, 1 $MgCl_2.6H_2O$, 1.3 Ca-Gluconate.H_2O) was supplemented with 10 mM Indomethacin to block the basal cAMP production and the pH was adjusted to 7.4. The concentrations of apical and basal Cl^- were maintained equivalent. After a 20-min equilibrium period, amiloride (20 μM Amil) was added to the luminal side to block ENaC-mediated Na^+ flow, then the cAMP agonist forskolin (2 μM FSK), the CFTR potentiator VX-770 (10 μM) (cells from patients P07 and P10 were not tested with VX-770), and the CFTR channel inhibitor (CFTInh172 – 30 μM) were added sequentially. All compounds were highest purity grade and obtained from Sigma-Aldrich, except VX-809 and VX-770 which were from SelleckChem.

2.4. DNA extraction

DNA was extracted by the phenol/chloroform method and for genetic analyses, a DNA concentration of 50 ng/mL was used as determined in a spectrophotometer (NanoVue™; GE Healthcare Biosciences, Pittsburgh, PA, USA).

2.5. Identification of the F508del mutation

The presence of the F508del mutation was determined by PCR with the primer pair [(5′-GGCACCATTAAAGAAAATATC-3′) and (5′-TGGCA TGCTTTGATGACGC-3′)], as previously described [36].

2.6. Determination of rs7512462 genotype in the SLC26A9 gene

Identification of rs7512462 genotype in the *SLC26A9* was performed by the TaqMan® SNP Genotyping system (Waltham, Massachusetts, USA) on a 7500 Real-Time PCR System® (Waltham, Massachusetts, USA), as previously [29].

2.7. Clinical data analysis

The clinical data used in this study included the sweat test (mmol/L), body mass index (BMI) and lung function markers achieved from spirometry (FEV_1; forced expiratory volume in 1 s of the FVC; FVC; forced vital capacity; FEV_1/FVC; $FEF_{25–75\%}$; forced expiratory flow at 25–75% of the pulmonary volume). All the data were collected from a database of a CF reference center. The sweat test was performed regarding the recently published guidelines and used the Gibson and Cooke method [37–40]. The spirometry was performed using as reference the instructions of American Thoracic Society and European Respiratory Society.

2.8. Statistical analyses

The descriptive analyses are presented by the mean ± SEM of all $\Delta I_{sc\text{-}eq}$. The $\Delta I_{sc\text{-}eq\text{-}FSK}$ was calculated by the subtraction of the $I_{sc\text{-}eq}$ generated by the $CFTR_{inh\text{-}172}$ (i.e., AMIL + FSK + VX-770 + $CFTR_{inh\text{-}172}$) by the $I_{sc\text{-}eq\text{-}FSK}$ (i.e. AMIL + FSK). The comparisons were made

Table 1
CFTR function in HNE cells from 12 F508del/F508del patients (P01-P12) and 2 controls (C01-C02 – wt/wt). Measurement of CFTR-mediated Cl^- secretion were obtained by the analysis of the mean and standard deviations of the deltas of equivalent short-circuit currents determined by stimulation of cAMP by FSK (2 μM) ($\Delta I_{sc\text{-}eq\text{-}FSK}$) and addition of the potentiator VX-770 (10 μM) to CRC-HNE incubated for 48 h with VX-809 (3 μM) or DMSO (vehicle) (0.05%).

Patient	$\Delta I_{sc\text{-}eq\text{-}FSK}$				p-Value (corrected p-value)#
	DMSO	VX-770	VX-809	VX-809 + VX-770	
Control (wt/wt)					
C01	4.522 ± 0.417	4.292 ± 0.179	4.432 ± 0.975	4.451 ± 0.883	–
C02	3.984 ± 0.452	3.069 ± 0.579	3.905 ± 0.690	2.792 ± 0.526	–
Mean	4.292 ± 0.301*	3.768 ± 0.344**	4.206 ± 0.592***	3.740 ± 0.612****	
F508del/F508del					
P01	0.059 ± 0.111#	0.110 ± 0.043	0.149 ± 0.086	0.211 ± 0.049#	0.223 (1)
P02	0.211 ± 0.054#	0.154 ± 0.050	0.442 ± 0.088#	0.382 ± 0.082	0.068 (0.816)
P03	0.150 ± 0.036#	0.199 ± 0.040	0.197 ± 0.009	0.365 ± 0.163#	0.267 (1)
P04	0.290 ± 0.167	0.400 ± 0.081	0.296 ± 0.137	0.437 ± 0.187	–
P05	0.187 ± 0.127#	0.128 ± 0.060	0.348 ± 0.122	0.381 ± 0.090#	0.233 (1)
P06	0.125 ± 0.012	0.145 ± 0.034	0.150 ± 0.077	0.129 ± 0.032	–
P07	−0.025 ± 0.019#	–	0.076 ± 0.080#	–	0.462 (1)
P08	0.080 ± 0.078#	0.187 ± 0.078	0.565 ± 0.117	0.610 ± 0.092#	**0.009** (0.108)
P09	0.274 ± 0.087	0.352 ± 0.135	0.803 ± 0.273	0.829 ± 0.244	0.223 (1)
P10	0.185 ± 0.033	–	0.364 ± 0.022	–	–
P11	0.452 ± 0.101#	0.626 ± 0.183	0.441 ± 0.077	0.487 ± 0.041#	0.732 (1)
P12	0.215 ± 0.056#	0.230 ± 0.086	0.491 ± 0.136	0.564 ± 0.011#	**0.004 (0.048)**
Mean	0.186 ± 0.028*	0.230 ± 0.034**	0.372 ± 0.048***	0.439 ± 0.049****	
p-Value (corrected p-value)	**< 0.001 (< 0.004)**	**< 0.001 (< 0.004)**	**< 0.001 (< 0.004)**	**< 0.001 (< 0.004)**	

Student's *t*-test: #: Comparison of the mean for VX-809 or VX-809 + VX-770 (higher) with the mean for DMSO in each individual patient; *Comparison of the DMSO treatment between the mean of the controls and the mean of the patients; **Comparison of the VX-770 treatment between the mean of the controls and the mean of the patients; ***Comparison of the VX-809 treatment between the mean of the control and the mean of the patients; ****Comparison of the VX-809 + VX-770 treatment between the mean of the controls and the mean of the patients. Positive p-value is shown in bold type (p-value < 0.05). The p-values are shown as uncorrected p-value (corrected p-value by multiple comparison – Bonferroni, test was applied). Cells from P07 and P10 were not tested with the potentiator VX-770.

using Student's *t*-test. The tests were two-tailed and the alpha of 0.05 was adopted in all analyzes.

The multiple-comparison correction by Bonferroni correction was done to be a conservative approach. In our data, we showed the uncorrected and corrected p-values. We considered as a significant p-value the follow data: (i) ≤0.05/12 representing the p-values achieved from the comparison of each patient (Table 1); (ii) ≤0.05/4 representing the p-values achieved from the comparison of patient's groups with control group to each intervention (Table 1); (iii) ≤0.05/12 representing the p-values achieved among the association of genotypes groups by rs7512462 (*SLC26A9*) and interventions (Table 2 and Fig. 1C); (iv) ≤0.05/36 representing the p-values achieved from the comparison of each patient and between groups (Fig. 1A); (v) ≤0.05/8 representing the p-values achieved from the comparison between wt/wt versus F508del/F508del patients and between the interventions (Fig. 1B); (vi) ≤0.05/9 representing the p-values achieved from the comparison between interventions in each genotypic group (Fig. 1C).

3. Results

3.1. Rescuing of CFTR function by VX-809/VX-770 in CRC-HNE cells from F508del/F508del patients is highly variable

CFTR-mediated Cl^- secretion was evaluated in CRC-HNE cells from 12 CF individuals with F508del/F508del (P01-P12) and 2 non-CF (C01-C02 – wt/wt). Rescue of CFTR function was quantified by the analysis of the deltas of equivalent short-circuit currents ($\Delta I_{sc\text{-}eq}$) determined by the stimulation of cAMP by FSK at baseline and after the addition of potentiator VX-770 after 48 h-incubation with corrector VX-809 and/or DMSO as vehicle control (Fig. 1; Table 1). The $\Delta I_{sc\text{-}eq\text{-}FSK}$ analyzed of the HNE cells from 2 non-CF individuals were combined for the normal CFTR function (4.292 ± 0.301 μA/cm^2–100%). The $\Delta I_{sc\text{-}eq\text{-}FSK}$ analyzed from HNE cells of CF patients, within all treatment's groups were evaluated in comparison to the non-CF CFTR function (100%) for the calculation of CFTR rescue by VX-809 + VX-770 (Fig. S1; Table S1).

Our data in HNE cells from F508del/F508del individuals evidenced

Table 2
CFTR function in HNE cells from 12 F508del/F508del (P01-P12) patients by rs7512462 (*SLC26A9*). Measurements of CFTR-mediated Cl^- secretion were obtained by the analysis of the mean and standard deviations of the deltas of equivalent short-circuit currents determined by the stimulation of cAMP by FSK (2 μM) ($\Delta I_{sc\text{-}eq\text{-}FSK}$) and the addition of the potentiator VX-770 (10 μM) to HNE cells incubated for 48 h with VX-809 (3 μM) or DMSO (vehicle) (0.05%).

	$\Delta I_{sc\text{-}eq\text{-}FSK}$			
	DMSO	VX-770	VX-809	VX-809 + VX-770
SNP rs7512462				
TT	0.166 ± 0.035	0.171 ± 0.026	0.261 ± 0.046	0.306 ± 0.045
CT	0.208 ± 0.057	0.362 ± 0.094	0.493 ± 0.091	0.656 ± 0.101
CC + CT	0.209 ± 0.047	0.329 ± 0.074	0.492 ± 0.080	0.639 ± 0.082
p-Value (corrected p-value)				
CT vs TT	0.5150 (1)	**0.0140** (0.1680)	**0.0201** (0.2412)	**0.0008 (0.0096)**
CC + CT vs TT	0.4617 (1)	**0.0223** (0.2676)	**0.0137** (0.1644)	**0.0004 (0.0480)**
CC + CT vs CT	0.9854 (1)	0.7828 (1)	0.9984 (1)	0.8934 (1)

p-Value: Student's *t*-test comparison for the TT, CT and CC + CT SNP groups. Positive p-value is shown in bold type (p-value < 0.05). The p-values are shown as uncorrected p-value (corrected p-value by multiple comparison – Bonferroni, test was applied).

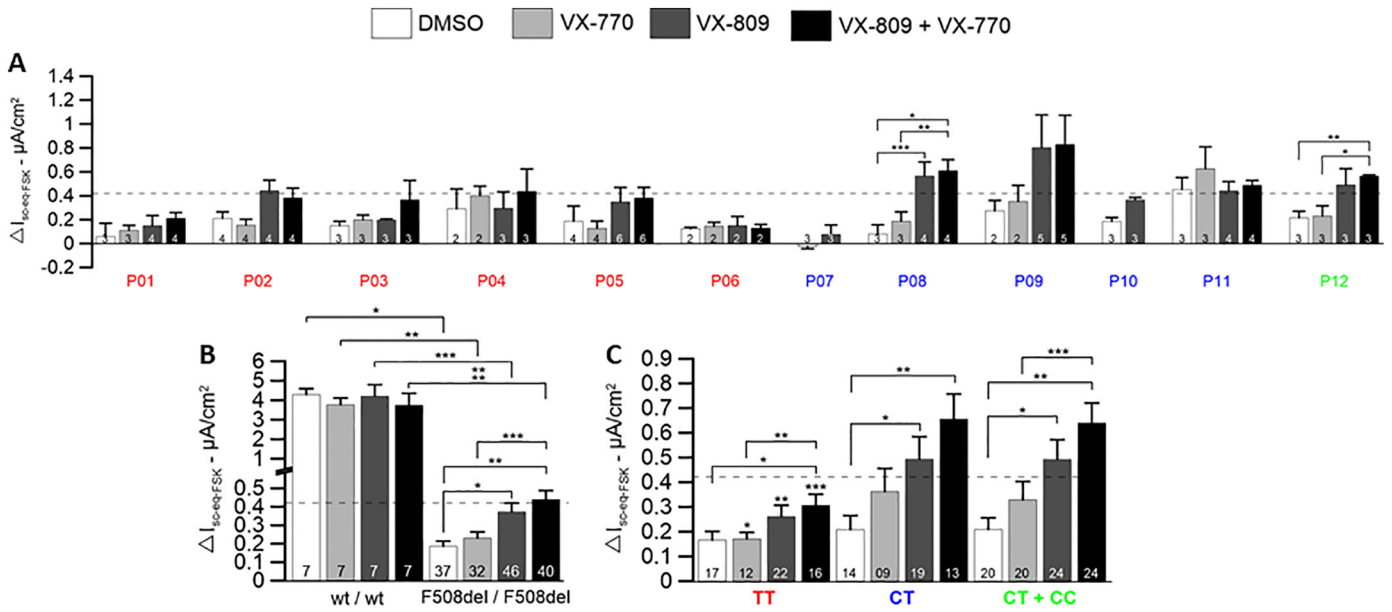

Fig. 1. Values of CFTR-mediated Cl^- secretion in CRC-HNE cells from 12 F508del/F508del patients and 2 non-CF individuals (wt/wt). Cells were analyzed by Ussing Chamber after treatment for 48 h at 37 °C with DMSO (0.05%) or VX-809 (3 μM) and analyzed in 4 groups: DMSO (white); VX-770 (light grey); VX-809 (dark grey); and VX-809 + VX-770 (black). (A) Individual values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ (2 μM) of CRC-HNE cells for all 12 patients. (B) Combined values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ in CRC-HNE cells from the 2 non-CF individuals (wt/wt) and from the 12 F508del/F508del patients. (C) Values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ in CRC-HNE cells from 12 F508del/F508del patients grouped by the rs7512462 genotype (*SLC26A9*): TT (red), CT (blue) and CC + CT (green). Horizontal lines indicate 10% of the CFTR function for the 2 non-CF individuals (wt/wt – 100%). Numbers inside the bars correspond to the number of filters analyzed. Significant different of Student's *t*-test (Bonferroni): (A) (P08) *0.025 (0.900); **0.021 (0.756); ***0.009 (0.324); (P12) 0.004 (0.144); **0.018 (0.348); (B) *0.001 (0.008); **0.001 (0.008); ***0.001 (0.008); ****0.001 (0.008); and *0.002 (0.018); **0.001 (0.008); ***0.001 (0.008); (C) TT: *0.022 (0.198), **0.018 (0.162); CT: *0.021 (0.189); **0.001 (0.009); CT + CC: *0.008 (0.072); **0.001 (0.009); ***0.012 (0.108); TT × CT: *0.014 (0.168); **0.020 (0.241); ***0.0008 (0.0096); TT × CT + CC: *0.022 (0.268); **0.014 (0.164); ***0.0004 (0.048). The p-values are shown as uncorrected p-value (corrected p-value by multiple comparison – Bonferroni, test was applied). Cells from P07 and P10 were not tested with the potentiator VX-770.

high variability in the basal CFTR function (i.e., under DMSO), presenting different values of $\Delta I_{sc\text{-}eq\text{-}FSK}$, as well as in the rescue of CFTR function in response to VX-770, VX-809 and VX-809 + VX-770. For example, P11 had the highest values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ (0.452 ± 0.101 μA/cm^2), in contrast to P07 which demonstrated the lowest $\Delta I_{sc\text{-}eq\text{-}FSK}$ (0.012 ± 0.251 μA/cm^2), and this occurred in all treatments (Fig. S1A). Indeed, for treatment with VX-770, P11 evidenced the highest values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ (0.626 ± 0.183 μA/cm^2) and P01 the lowest (0.110 ± 0.043 μA/cm^2). On the other hand, P09 had the highest values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ in response to either VX-809 alone (0.803 ± 0.273 μA/cm^2) or VX-809 + VX-770 (0.829 ± 0.244 μA/cm^2), while P07 had the lowest $\Delta I_{sc\text{-}eq\text{-}FSK}$ with VX-809 (0.076 ± 0.080 μA/cm^2) and P06 had the lowest $\Delta I_{sc\text{-}eq\text{-}FSK}$ with VX-809 + VX-770 (0.129 ± 0.032 μA/cm^2, Fig. S1A). Interestingly, P11 demonstrated a small negative modulation of CFTR function by VX-809 vs VX-770 (not significant) and P12 was the only patient who had significantly higher values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ in the presence of VX-809 + VX-770 compared with the DMSO and VX-770 (Figs. 1A, 2; Table 1).

Grouping all the analyses performed for the CRC-HNEs from F508del/F508del individuals, treatments with VX-809 alone and VX-809 + VX-770 evidenced a significant increase in the values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ in comparison with the control (DMSO). Similarly, values for $\Delta I_{sc\text{-}eq\text{-}FSK}$ of VX-770 + VX-809 were significantly higher than those for VX-770 alone ($p < 0.05$) (Fig. 1B). Furthermore, and as expected, this group had values of basal $\Delta I_{sc\text{-}eq\text{-}FSK}$ significantly lower than those of the control group (C01 + C02 – wt/wt), either in the presence of the drugs or not ($p < 0.05$) (Fig. 1B; Table 1). Rescue of CFTR function in CRC-HNEs from individuals with CF corresponded to CFTR function of 5.36% (± 0.80) with VX-770, 8.66% (± 1.11) after incubation with VX-809, and of 10.23% (± 1.15) after the combination treatment, i.e., VX-809 + VX-770 (Fig. S1B, Table S1).

Regarding levels of CFTR function, both values for $\Delta I_{sc\text{-}eq\text{-}FSK}$ basal (DMSO) and those for rescue by VX-770 remained below 10% of control CFTR currents exhibited by the wt/wt cells (100%). The only exception was P11 that presented basal values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ above 10%. After VX-809 treatment, CRC-HNEs from P02, P08, P09, P11 and P12 demonstrated values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ higher than 10% of wt/wt function and in the presence of VX-809 + VX-770 the values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ of the CRC-HNEs from P04, P08, P09, P11 and P12 were higher than 10%. The CRC-HNEs from the P01, P03, P04, P05, P06, P07 and P10 tested with VX-809 and P01, P02, P03, P05 and P06 tested with VX-809 + VX-770 had values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ below 10% of non-CF cells (Figs. 1A, 2, S1A; Table S1).

3.2. The SNP rs7512462 in SLC26A9 genotype correlate with the extent of F508del-CFTR rescue by VX-809/VX-770

In order to determine whether the above variability correlated with the SNP rs7512462 in *SLC26A9*, the values obtained for the currents in the CRC-HNEs from the 12 patients under study were grouped according to the 3 possible genotypes of this SNP (i.e., CC, CT and TT) as follows: 6 TT (P01, P02, P03, P04, P05 and P06); 5 CT (P07, P08, P09, P10, P11) and 1 CC (P12) (Figs. 1C, 2, S1C). Values of CFTR function ($\Delta I_{sc\text{-}eq\text{-}FSK}$) under the 4 conditions (DMSO; VX-770; VX-809 and VX-809 + VX-770) were compared among those 3 SNP groups, namely: (i) TT vs CT; (ii) TT vs CT + CC. This latter group grouped two genotypes because there was only one individual with CC. For CRC-HNEs without corrector (i.e., incubated with DMSO), the CT and the CT + CC groups presented higher values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ in the presence of VX-770 than those in the TT group. The same was observed after incubation with VX-809 and VX-809 + VX-770, being the values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ of the CT and CT + CC groups higher than those of the TT group ($p < 0.05$) (Fig. 1A, Table 1). In addition, the CT and CT + CC groups had significantly

higher values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ with VX-809 than under DMSO, while the TT group had not; all groups had higher values had significantly higher values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ for VX-809 + VX-770 than DMSO; and finally the CT + CC group had significantly higher $\Delta I_{sc\text{-}eq\text{-}FSK}$ for VX-809 + VX-770 than VX-770 ($p < 0.05$) (Fig. 1A, Table 1).

As to CFTR function rescued by the VX-809 + VX-770 combination, the CT (15.29% ± 2.35) and CT + CC (14.89% ± 1.91) groups evidenced values which were higher than 10% of the wt/wt function (100%), which was not observed for the TT (7.13% ± 1.05) group (Figs. 1C, 2B, D, F, S1C).

The rs7512462*C allele thus seems to exert a positive effect on F508del-CFTR function while rs7512462*T a negative one, as indeed

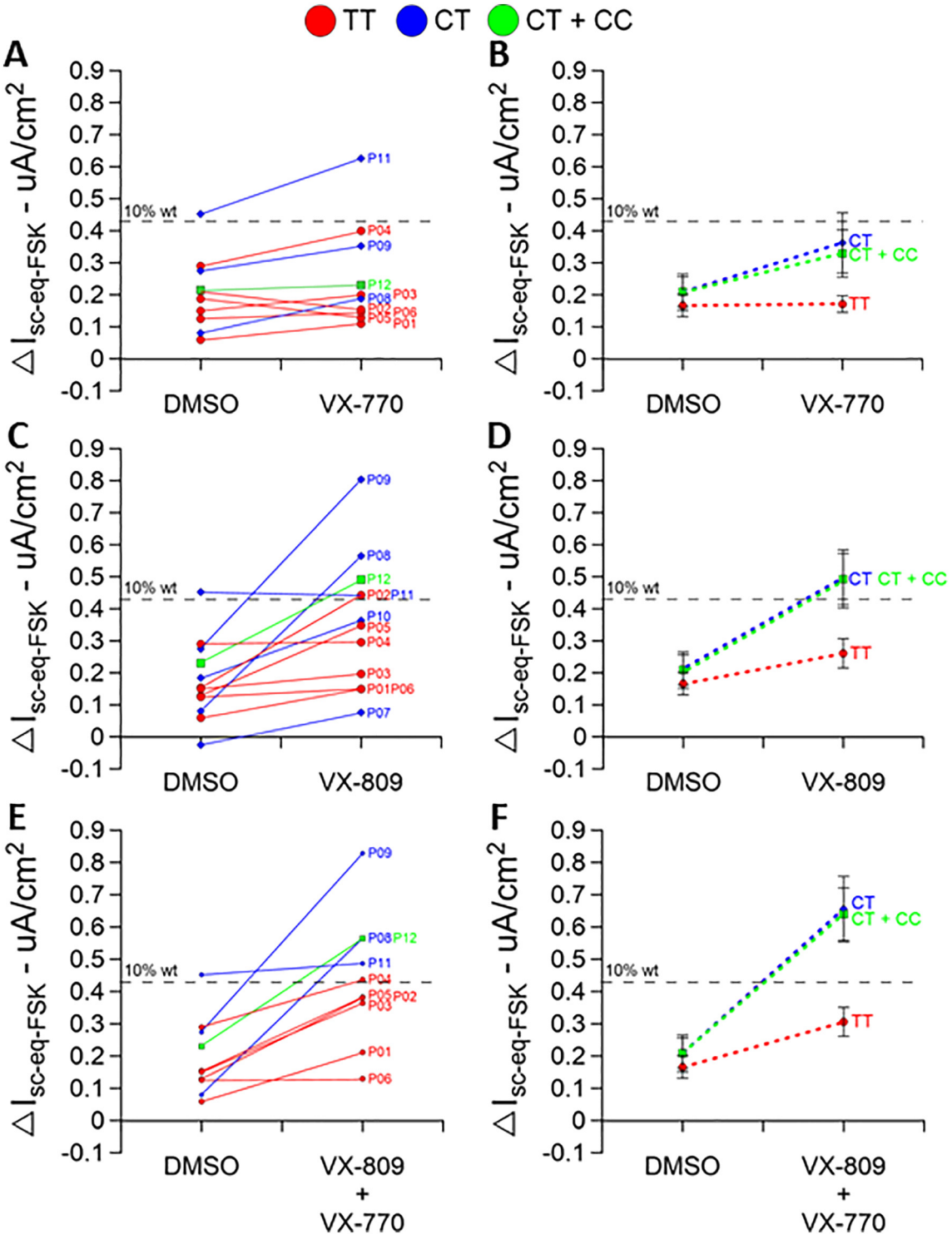

Fig. 2. Rescue of CFTR function by the CFTR modulator drugs VX-770 (A, B), VX-809 (C, D) or the VX-809 + VX-770 combination (E,F) according to the rs7512462 genotype (*SLC26A9*). Individual shifts in the values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ (2 μM) in CRC-HNE cells from each of the 12 F508del/F508del patients according to the rs7512462 genotype (*SLC26A9*): TT (red); CT (blue); CC + CT (green), after treatment with A. VX-770, C. VX-809 and E. VX-809 + VX-770. Shifts in the values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ (2 μM) all individuals grouped by the rs7512462 (*SLC26A9*) genotype: B. VX-770, D. VX-809 and F. VX-809 + VX-770. Horizontal lines indicate 10% of the CFTR function for the 2 non-CF individuals (wt/wt – 100%). Cells from P07 and P10 were not tested with the potentiator VX-770.

values for $\Delta I_{sc\text{-}eq\text{-}FSK}$ with VX-809 and VX-809 + VX-770 were significantly higher for rs7512462*C, when compared to those for rs7512462*T ($p < 0.05$). Also, both CT + CC and CT groups had evidenced significant higher values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ in the groups VX-809 and VX-809 + VX-770 in comparison with DMSO and VX-770 (Fig. S2A). Furthermore, the rs7512462*C allele (14.61% ± 1.60), correlated with higher levels of F508del-CFTR function rescue by both VX-809 and VX-770, than the rs7512462*T allele (8.87% ± 0.87) (Fig. S2B, Table S1).

3.3. SNP rs7512462 (SLC26A9) evidence a correlation in the clinical data analysis with basal F508del–CFTR function in CRC-HNE

Clinical data of 12 F508del/F508del individuals were compared with the values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ (i.e., CFTR function with DMSO). The sweat Cl^- levels in these individuals evidenced a small correlation with the basal CFTR function ($\Delta I_{sc\text{-}eq\text{-}FSK}$ values) in relation to SNP rs7512462. The TT group, which presented a lower mean of $\Delta I_{sc\text{-}eq\text{-}FSK}$ values (0.166 ± 0.035 μA/cm^2) had the highest levels of sweat Cl^- (109.19 ± 13.18 mmol/L) and CT and CC + CT groups, which had higher mean of $\Delta I_{sc\text{-}eq\text{-}FSK}$ values (0.208 ± 0.057 μA/cm^2; 0.209 ± 0.047 μA/cm^2; respectively), had lower levels of sweat Cl^- (105.01 ± 6.79 mmol/L; 101.90 ± 6.36 mmol/L, respectively) (Fig. 3A, B, Table S3). Similar correlations were also evidenced with BMI of those 12 CF individuals in relation to the SNP rs7512462, with the TT group showing lower values of both BMI (16.38 ± 0.67 Kg/m^2) and $\Delta I_{sc\text{-}eq\text{-}FSK}$, and the CT (17.28 ± 1.29 Kg/m^2) and CC + CT (17.53 ± 1.08 Kg/m^2) groups evidencing higher BMI and $\Delta I_{sc\text{-}eq\text{-}FSK}$ (Fig. 3C, D, Table S2).

These positive correlations of $\Delta I_{sc\text{-}eq\text{-}FSK}$ values with sweat test and BMI were not observed for lung function (spirometry analyses, predicted values), that showed a higher lung function, expressed by FEV_1 (80.25 ± 13.33), FVC (89.25 ± 13.30) or FEV_1/FVC (88 ± 4.53) in the TT group (with lower $\Delta I_{sc\text{-}eq\text{-}FSK}$) and lower lung function in the CT (FEV_1: 69.40 ± 3.03; FVC: 83.20 ± 3.88; FEV_1/FVC: 78.80 ± 1.71) and CC + CT groups (FEV_1: 69.67 ± 2.49; FVC: 82.67 ± 3.21; FEV_1/FVC: 81.17 ± 2.75) which also have higher $\Delta I_{sc\text{-}eq\text{-}FSK}$ values (Figs. 3E, F, S3, Table S3).

4. Discussion

It was previously demonstrated that CFTR regulates several other epithelial ion channels and transporters, namely ENaC [41,42], renal outer medullary potassium channel (ROMK) [43], solute carrier family 26 member 4 (SLC26A4) [44], SLC26A9 [31], transmembrane member 16A/anoctamin-1 (TMEM16A/ANO1) [45], among others. In turn, some of these were also shown to modulate the expression and function of CFTR [46–48]. This relationship may be useful to clarify the high diversity in CF phenotypes among patients with the same *CFTR* genotype as well as with highly variable responses to CFTR modulator drugs [29,30].

In the present study, we measured the basal CFTR function ($\Delta I_{sc\text{-}eq\text{-}FSK}$) and the respective responses to CFTR modulator drugs in HNE cells from individuals with CF who are F508del-homozygous. We found that values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ are highly variable (Figs. 1A, S1A) consistently with the variability of their clinical phenotypes and also with the heterogeneity of the responses reported for these drugs [22–24]. We hypothesized that such variability could, at least partially, result from the contribution of modifying genes, in addition to the respective *CFTR* genotype and of course also environmental factors.

In an attempt to elucidate the function and response to CFTR drugs in CF patients with the same *CFTR* genotype, several studies have evaluated how modifier genes and their expression/variability, can influence both CFTR expression and CF clinical phenotypes [27–31]. The analysis of allelic variants in several modifying genes that modulate CFTR function and expression in the epithelium of different CF-targeted organs should be emphasized. Against this background, we and others have previously demonstrated the impact of modifier gene variants of alternative ion channels on the variability both of CF clinical phenotypes and response to CFTR modulator drugs [16,27–31]. Since CFTR expression and regulation is organ dependent, prediction of drug efficacy should be performed in cells which are physiologically relevant to the main organ affected in CF and most associated with mortality and morbidity i.e., the airways. Therefore, CRC-HNE cells were selected for the present study because they are easier to obtain and have been shown to produce similar results to those in HBE cells, the gold standard in CF research [49,50].

Our data measuring CFTR function ($\Delta I_{sc\text{-}eq\text{-}FSK}$) in CRC-HNE cells show that rs7512462 (*SLC26A9*) correlates with basal CFTR function in these cells from F508del/F508del patients. The rs7512462*C allele had a positive correlation (higher function), and rs7512462*T, a negative correlation with CFTR function (lower function, Fig. S2). Individuals who had the *SLC26A9* rs7512462*CT or rs7512462*CC genotypes had levels of basal CFTR function and of response to VX-809 or VX-770 in CRC-HNEs which were significantly higher than those with rs7512462*TT (Figs. 1A, C, 2, S1A, C). These data corroborate findings in HBE cells [30], demonstrating that HNE cells are a useful tool to study CFTR function and response to CFTR modulator drugs. In addition, individuals with CT genotype and CT + CC group for the SNP rs7512462 evidenced CFTR responses to VX-809 and VX-770 which were > 10% of the function of wt/wt HNE cells. These findings appear to indicate that F508del/F508del patients who have rs7512462*C allele at *SLC26A9*, could benefit more from VX-809 + VX-770 (Orkambi®) than those with rs7512462*T, being thus expected a greater improvement in disease symptoms, namely in the airways. Despite that we only analyzed a limited number of patients here (n = 12) and we have no clinical data for patients under treatment, these data are suggestive that a 10% increase in CFTR function in HNEs may correspond to an outcome for the patient that brings the classical form of the disease to a milder, non-classical CF phenotype, since it was postulated that CFTR function between 10 and 25% can at least significantly delay the progression of pulmonary disease [50,51].

Allying these lines, we also compared the basal CFTR function in CRC-HNE with the latest performed clinical tests on patients (sweat test, BMI and lung function) according to the allelic variant of the SNP rs7512462 in *SLC26A9*. In these comparisons the values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ correlated with sweat [Cl^-], with CC + CT and CT groups having higher values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ and lower [Cl^-] and the TT lower $\Delta I_{sc\text{-}eq\text{-}FSK}$ and higher [Cl^-] in sweat (Fig. 3A, B). However, this correlation was not significant and more experiments are required in patients with the same *CFTR* and *SLC26A9* genotypes to prove this correlation. Furthermore, to date expression of the SLC26A9 protein has not been reported to occur in the sweat gland.

Similarly, the comparison of the BMI of the CF patients under study with the levels of CFTR function in CRC-HNE according to the allelic variant of the SNP rs7512462 in *SLC26A9* also showed a positive correlation, evidencing higher values of BMI and $\Delta I_{sc\text{-}eq\text{-}FSK}$ in the CC + CT and CT groups (i.e., those with higher values of $\Delta I_{sc\text{-}eq\text{-}FSK}$), and lower in the TT group, which also had lower $\Delta I_{sc\text{-}eq\text{-}FSK}$ values (Fig. 3C, D). It is likely that these differences have physiological meaning, since SLC26A9 is expressed in the intestine, stomach and lung, so it is expected that its function may be modulated by the SNP which could possibly modulate the interaction of this channel with CFTR, thus impacting on the CF phenotype in these organs, which would translate into BMI [52–54].

Nevertheless, there was no correlation of $\Delta I_{sc\text{-}eq\text{-}FSK}$ with the pulmonary function (measured by spirometry tests FEV_1, FVC and FEV_1/FVC), being observed in contrast higher pulmonary functions in the group of patients with the *SLC26A9* rs7512462*TT genotype (i.e., the one with lower values of $\Delta I_{sc\text{-}eq\text{-}FSK}$), than in CT + CC and CT groups, which had higher $\Delta I_{sc\text{-}eq\text{-}FSK}$ (Figs. 3C, D, S3). It should be mentioned that recently spirometry has had a progressive loss of value in assessing

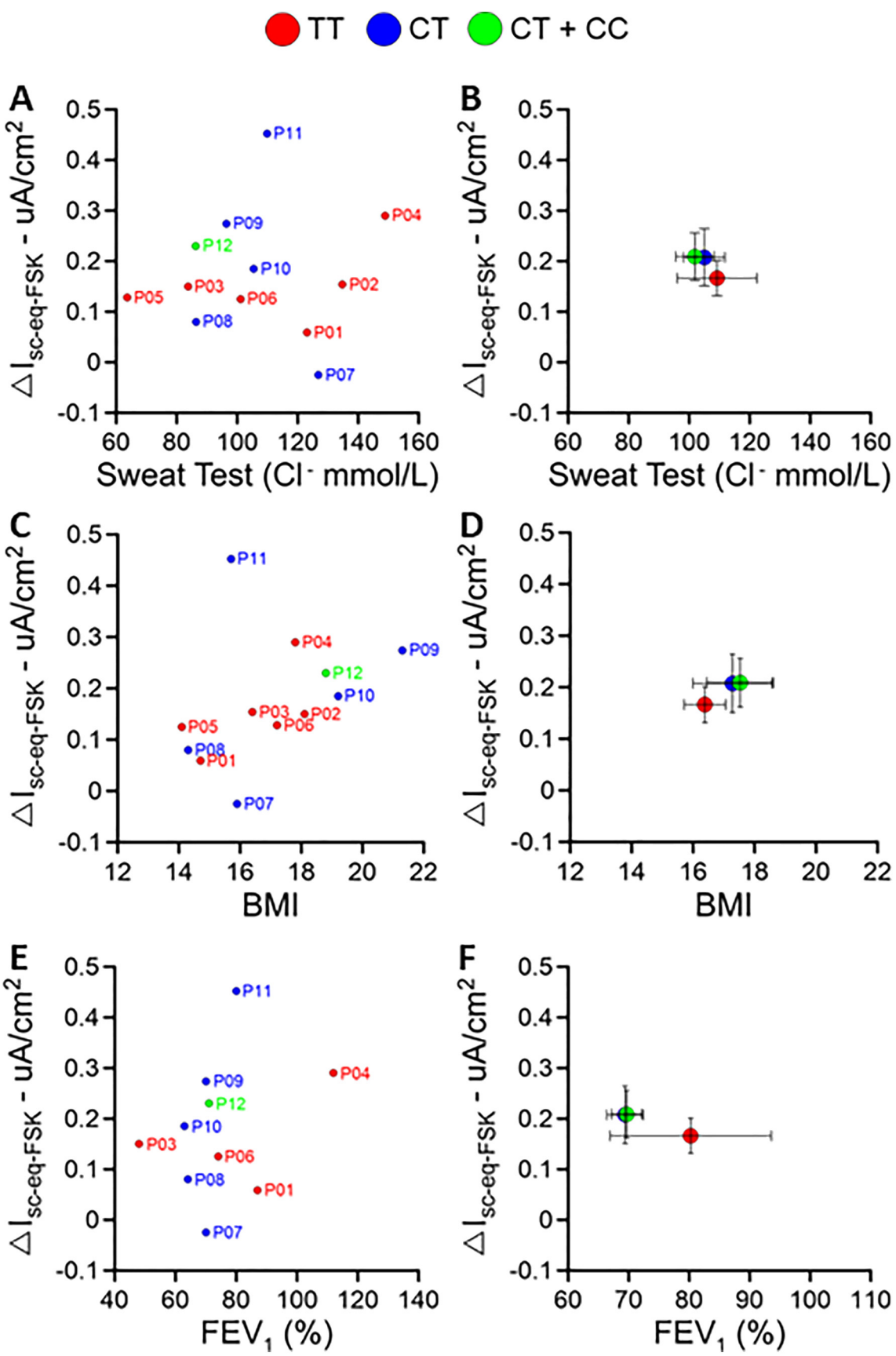

Fig. 3. Comparison of clinical parameters of 12 F508del/F508del patients with basal CFTR-mediated Cl^- secretion in CRC-HNE cells grouped by the rs7512462 genotype (*SLC26A9*). The distinct rs7512462 genotypes are represented in red (TT), blue (CT) and green (CC + CT), respectively. Individual values of $\Delta I_{sc\text{-}eq\text{-}FSK}$ (2 μM) in CRC-HNE cells from the 12 F508del/F508del patients for each patient individually (A, C, E), or all patients grouped by the rs7512462 genotype (B, D, F), were correlated with: (A, B) sweat test ([Cl^-] = mmol/L); (C, D) body mass index (BMI); (E, F) maximum forced expiratory volume in 1 s (FEV_1).

lung function in several respiratory diseases, namely in CF, due to the small difference that these tests present in the interventions with the CFTR modulating drugs. The correlation of spirometry measurements in clinical trials has presented divergent correlations vs those obtained for other parameters, such as BMI, lung clearance index (LCI) and sweat [Cl^-] [55–58]. Moreover, in a previous study [51] where we did find a positive correlation between the levels of CFTR function in rectal biopsies with lung function (FEV_1), the number of patients in the cohort was much larger (n = 118) thus allowing age stratification, while here the much lower number of patients analyzed here (n = 12) did not allow such stratification. Other authors studying a much larger cohort of F508del/F508del patients also failed to show a correlation between the lung function of F508del/F508del patients and CFTR function in HBE cells according to the SNP of the *SLC26A9* gene [30]. However, those authors did not perform age stratification of the patients. We thus believe that more patients should be evaluated for CFTR function in CRC-HNEs and correlations established with pulmonary function data according to age-dependent stratification for a better analysis of interaction of the SNPs *SLC26A9* and pathogenic variants in the *CFTR*.

The comparison of levels of CFTR function with clinical data (and changes thereof under drug treatments) are of great importance for CF as good in vitro models that reliably predict clinical patient benefit for novel CFTR modulator are very much in need [49]. Despite that In the present study, the in vitro data could not be correlated with clinical improvement because the patients at our center are not yet under treatment by these drugs (Ivacaftor® and Orkambi®) the good correlation of basal CFTR function determined in CRC-HNE and at least some of the clinical parameters analyzed (sweat Cl^- and BMI) lead us to suggest that this is a good predictive model.

Our study was the first to focus on the range of values obtained for F508del-CFTR function measured in HNE cells and its correlation with allelic variants in a CFTR modifying gene (*SLC26A9*). Our findings clearly suggest that HNE cells constitute a useful and relatively easy to use in vitro model for the practice of personalized medicine, namely to test CFTR modulating drugs, and help making decisions on the best drug (or combination of drugs) which best rescues CFTR function in the individual's own cells (Figs. 1, 2, S1, S2) [7,20–26,49,50]. Indeed, this study demonstrates the technical feasibility of the CRC-HNE cells as a reliable in vitro cellular model, making it a useful tool to test these new drugs in rescuing rare (orphan) *CFTR* mutations, with similar results to those in HBE cells [49,50] but far less invasive to obtain.

Moreover, due to the wide inter-individual variability of outcomes reported for the novel CFTR modulator drugs, measurement of CFTR functional response in CRC-HNE cells allows the experimental individual validation of drug efficacy, already taking into account the multiple positive/negative effects of all modifying genes.

Nevertheless, prospects for assessing accuracy in CF precision medicine using this model to test efficacy of CFTR modulator drugs will still include: (i) validating findings in other *CFTR* genotypes (i.e., F508del heterozygotes), as a large group of patients with a possible benefit from the use of those drugs; (ii) validate the findings using additional CFTR modulator drugs, such as VX-661-VX-770 (Tezacaftor®), in HNE cells from F508del/F508del patients; (iii) validate the findings for CFTR function in HNE cells with other biomarkers, including intestinal organoids [59,60] which may reflect the specificity of each organ; (iv) include multiple modifying genes in the analysis of CFTR function and drug response, namely those associated with alternative ion channels already found to influence severity of CF, based on genome wide approach association studies; (v) identify the responsible genes and mechanism by which CF patients with the same *CFTR* genotype, present variable levels of residual CFTR function and include these genes in a possible strategy for correction of CFTR function; (vi) validate these experimental in vitro findings by correlating them with clinical parameters from CF patients under treatment with these drugs; (vii) compare levels of CFTR function in CRC-HNE with those obtained other biomarkers, namely intestinal organoids and clinical data for CF patients under treatment to find the best predictors.

As limitations of the use of the CRC-HNE cells as model system in our current study, we include: (i) small sample size, despite that our yields are in accordance with the previously published ones, the amount of cells collected and the low number of passages they allow, still makes this as a limited resource, in contrast e.g., with intestinal organoids which can be grown indefinitely; (ii) the current data for CFTR modulator drugs could not be clinically validated due to the absence of clinical data on the outcome of drug treatment; (iii) the current data only applied to F508del/F508del patients, despite this being the most prevalent mutation.

5. Conclusion

We can conclude from the current study that in our cohort, the CFTR functional response to VX-809 and VX-770 in CRC-HNE cells from F508del/F508del patients is conditioned by the *SLC26A9* allelic variants at the SNP rs7512462. Thus, restoration of the CFTR function by these, or other, drugs is also likely to be influenced by multiple other genes, especially of alternative ion channels. In order to evaluate the individuality of each patient and drug response in precision medicine we may resource to CRC-HNEs as a good in vitro model of CFTR function in the airways.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.01.029.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Competing financial interests

None.

Author contribution

AK, FALM and MDA designed the research. AK, FALM, SVP, AMV and GSL performed experiments. AK, FALM analyzed data. AK, FALM and MDA wrote the manuscript. JDR, AFR and CSB revised the manuscript.

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