



OPEN ACCESS

EDITED BY

Sabine Fuchs,
University Medical Center Utrecht,
Netherlands

REVIEWED BY

Tsukasa Okiyoneda,
Kwansei Gakuin University, Japan
Jiunn-Tyng Yeh,
National Yang Ming Chiao Tung
University, Taiwan

*CORRESPONDENCE

Ines Pankonien,
✉ ipankonien@ciencias.ulisboa.pt
Margarida D. Amaral,
✉ mdamaral@ciencias.ulisboa.pt

†PRESENT ADDRESS

Iris A. L. Silva and Jan Bartosch, Portugal
(IALS) and Institute of Clinical
Neuroimmunology, Biomedical Center of
the Ludwigs Maximilian University of
Munich, Munich, Germany

†These authors have contributed equally
to this work

SPECIALTY SECTION

This article was submitted to Molecular
Diagnostics and Therapeutics,
a section of the journal
Frontiers in Molecular Biosciences

RECEIVED 31 January 2023

ACCEPTED 06 March 2023

PUBLISHED 17 March 2023

CITATION

Railean V, Rodrigues CS, Ramalho SS,
Silva IAL, Bartosch J, Farinha CM,
Pankonien I and Amaral MD (2023),
Personalized medicine: Function of CFTR
variant p.Arg334Trp is rescued by
currently available CFTR modulators.
Front. Mol. Biosci. 10:1155705.
doi: 10.3389/fmolb.2023.1155705

COPYRIGHT

© 2023 Railean, Rodrigues, Ramalho,
Silva, Bartosch, Farinha, Pankonien and
Amaral. This is an open-access article
distributed under the terms of the
[Creative Commons Attribution License
\(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution or
reproduction in other forums is
permitted, provided the original author(s)
and the copyright owner(s) are credited
and that the original publication in this
journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Personalized medicine: Function of CFTR variant p.Arg334Trp is rescued by currently available CFTR modulators

Violeta Railean[†], Cláudia S. Rodrigues[†], Sofia S. Ramalho,
Iris A. L. Silva[†], Jan Bartosch[†], Carlos M. Farinha, Ines Pankonien*
and Margarida D. Amaral*

BioISI—Biosystems and Integrative Sciences Institute, Faculty of Sciences, University of Lisboa, Lisboa, Portugal

Most of the 2,100 CFTR gene variants reported to date are still unknown in terms of their disease liability in Cystic Fibrosis (CF) and their molecular and cellular mechanism that leads to CFTR dysfunction. Since some rare variants may respond to currently approved modulators, characterizing their defect and response to these drugs is essential for effective treatment of people with CF (pwCF) not eligible for the current treatment. Here, we assessed how the rare variant, p.Arg334Trp, impacts on CFTR traffic and function and its response to existing CFTR modulators. To this end, we performed the forskolin-induced swelling (FIS) assay on intestinal organoids from 10 pwCF bearing the p.Arg334Trp variant in one or both alleles of the CFTR gene. In parallel, a novel p.Arg334Trp-CFTR expressing CFBE cell line was generated to characterize the variant individually. Results show that p.Arg334Trp-CFTR does not significantly affect the plasma membrane traffic of CFTR and evidences residual CFTR function. This CFTR variant is rescued by currently available CFTR modulators independently of the variant in the second allele. The study, predicting clinical benefit for CFTR modulators in pwCF with at least one p.Arg334Trp variant, demonstrates the high potential of personalized medicine through theranostics to extend the label of approved drugs for pwCF carrying rare CFTR variants. We recommend that this personalized approach should be considered for drug reimbursement policies by health insurance systems/national health services.

KEYWORDS

cystic fibrosis, R334W, theranostics, personalized medicine, therotyping, organoids

1 Introduction

Cystic Fibrosis (CF) is the most common life-shortening autosomal recessive disorder among Caucasians, estimated to affect ~52,000 individuals in Europe and ~105,000 individuals worldwide (ECFS, 2020; Guo et al., 2022). CF is caused by variants in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, a cAMP-regulated chloride (Cl⁻) and bicarbonate (HCO₃⁻) channel expressed at the apical plasma membrane (PM) of epithelial cells (Riordan et al., 1989; Sheppard and Welsh, 1999). Defective CFTR function leads to abnormal ion transport causing dehydration of the mucus lining of mucosal surfaces, among those, the lung and the intestine (Rowe et al., 2005).

To date, about 2,100 alterations have been described in the CFTR gene (CFMDB, 2021), but the disease liability is only established for less than a quarter of these (CFTR, 2023). These variants have been grouped into seven functional classes according to their molecular/cellular defect and these are now evolving into theratypes (De Boeck and Amaral, 2016). Indeed, the underlying concept is that variants within the same class are rescued by the same therapeutic strategy, if not by the same drug (Cutting, 2015). Although this classification in theratypes is helpful, the significance of most variants is still unknown, not only regarding disease liability, but also the respective underlying molecular/cellular defect (De Boeck and Amaral, 2016; Sabusap et al., 2016). The most common CFTR variant is the deletion of three base pairs that result in the loss of phenylalanine at position 508: p.Phe508del (legacy name: F508del) (De Boeck and Amaral, 2016; CFMDB, 2023; CFTR, 2023). This variant causes CFTR misfolding leading to retention in the endoplasmic reticulum (ER) by the ER quality control (ERQC), premature degradation and failure to reach the PM (Ramalho et al., 2021). In addition, the p.Phe508del variant has also been shown to have a gating defect characterized by a reduction in open probability (Po) resulting in an abnormal Cl⁻ transport (Dalemans et al., 1991). Identification of small molecules that rescue the p.Phe508del-CFTR folding/trafficking/gating defect lead to the approval of three different corrector drugs currently available for individuals with CF bearing this mutation in combination with ivacaftor (iva), a CFTR gating potentiator: lumacaftor (luma), tezacaftor (teza) or elexacaftor (elexa, combined with teza) (Van Goor et al., 2006; Jih and Hwang, 2013; Wainwright et al., 2015; Taylor-Cousar et al., 2017; Middleton et al., 2019). These drugs are variant-specific and accordingly, they have been developed for pwCF homozygous for p.Phe508del (luma/iva; teza/iva; and elexa/teza/iva) or bearing at least one p.Phe508del (elexa/teza/iva). Consequently, most pwCF who have rare variants are not eligible for these treatments, except for those with gating mutations (class III) which are likely to be responsive to the potentiator (iva) alone.

However, some of the non-eligible rare variants are likely to respond to approved CFTR modulators (De Boeck and Amaral, 2016; Sabusap et al., 2016; Ramalho et al., 2021). Moreover, it is also often found that pwCF with the same CFTR genotype have significantly different clinical responses to CFTR-modulating drugs (Awatade et al., 2015; Wainwright et al., 2015). There is thus an unmet need to test these novel CFTR modulators directly *ex vivo* on the individual's tissues and/or primary cells with robust assays assessing modulator efficacy for a given CFTR genotype and thus predict clinical benefit for the donor individual.

One such rare variant, which is not included in the list of eligible variants for current CFTR modulators, is p.Arg334Trp. This is a missense variant which is located in transmembrane segment 6 (TM6) in the membrane spanning domain (MSD) one and characterized by decreased CFTR channel function not affecting channel folding or trafficking (Sheppard et al., 1993; Han et al., 2018). Previous studies have shown that the impairment is most likely caused by decreased conductance rather than Po since the treatment of p.Arg334Trp-CFTR expressing CFBE cells with potentiator iva only partly/slightly corrected its gating defect, i.e., to 5% of wt-CFTR transport activity (Veit et al., 2020). As such, p.Arg334Trp is included in class IV of CFTR variants (Sheppard et al., 1993). Moreover, in a different study it has also been observed that p.Arg334Trp-CFTR retained significant apical

membrane Cl⁻ channel function. Clinically, individuals carrying p.Arg334Trp-CFTR are more likely to be pancreatic sufficient and often present atypical CF disease (CFTR, 2023).

Here, we focus on determining the effect of the rare p.Arg334Trp variant on CFTR traffic (processing) and function and its response to existing CFTR modulators in more physiological relevant cell models. To this end, we used intestinal organoids from 10 individuals carrying the p.Arg334Trp variant in one or both alleles of the CFTR gene. In parallel, we generated a human CF bronchial epithelial cell line (CFBE41o⁻) expressing CFTR with this variant in order to study this variant separately/independently from other variants.

2 Materials and methods

2.1 Chemicals and compounds

All chemicals were of analytical grade. DMSO, Forskolin and Genistein were obtained from Sigma Aldrich, ivacaftor, tezacaftor and elexacaftor from Selleckchem (United States), and CFTR-inh172 from MedChemexpress (United States). All compounds were dissolved in DMSO.

2.2 CF subjects and ethical approval

Rectal biopsies were obtained from 10 Portuguese individuals with CF carrying the p.Arg334Trp CFTR variant. One of these was homozygous for p.Arg334Trp and the other nine had the following variants in their second CFTR allele: p.Arg1162X, p.Gly542X, p.Tyr1092X, p.Phe508del (4 individuals), p.Gln1100Pro, p.Thr1086Pro (c.3256A>C). Organoids from one Portuguese individual with the p.Phe508del/p.Phe508del genotype were used as control. Informed consent was obtained from all the subjects and the study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Hospital de Santa Maria, Lisboa, Portugal (DIRCLN-16JUL 2014–211). The individuals mean age was 34 years (interval: 26–46 years).

2.3 Human intestinal organoids and forskolin-induced swelling (FIS) assay

Crypt isolation from rectal biopsies, organoids culture and FIS assay were performed as described by Vonk et al. (Vonk et al., 2020). Organoids were treated with 0.02; 0.128; 0.8; and 5 μM of forskolin (fsk) alone or together with: only 3.33 μM iva; 5 μM teza +3.33 μM iva; or 3 μM elexa + 5 μM teza +3.33 μM iva (Vonk et al., 2020). FIS quantification was performed using Cell Profiler and Organoids Analyst Software (Hagemeyer et al., 2021), and the area under the curve (AUC) was calculated using GraphPad Prism 9.0.

2.4 Cell lines

Cell lines expressing wt-CFTR and p.Arg334Trp-CFTR were generated by lentiviral transduction of the human bronchial CFBE41o⁻ cell line (which does not express any endogenous

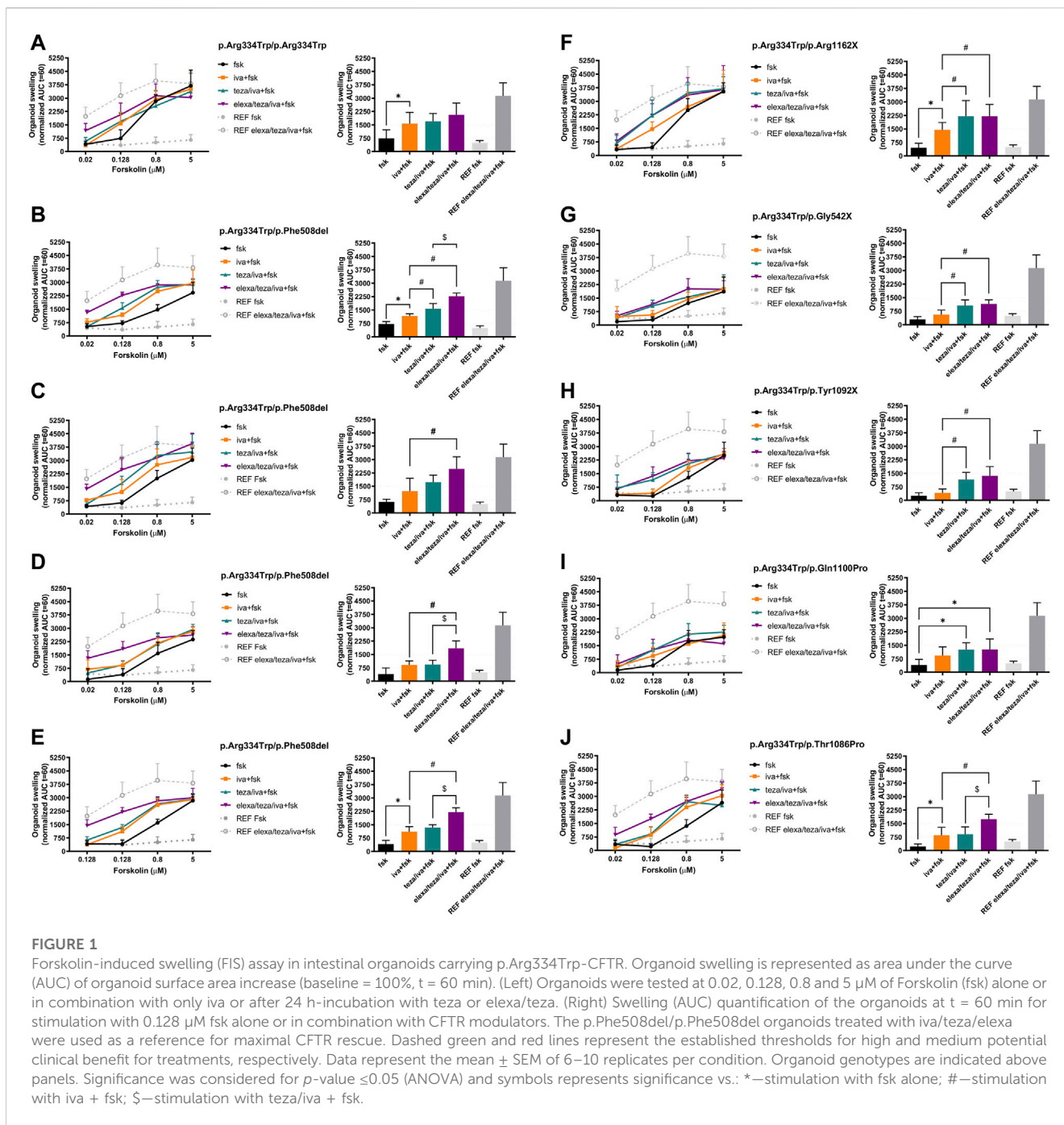


FIGURE 1

Forskolin-induced swelling (FIS) assay in intestinal organoids carrying p.Arg334Trp-CFTR. Organoid swelling is represented as area under the curve (AUC) of organoid surface area increase (baseline = 100%, t = 60 min). (Left) Organoids were tested at 0.02, 0.128, 0.8 and 5 µM of Forskolin (fsk) alone or in combination with only iva or after 24 h-incubation with teza or elexa/teza. (Right) Swelling (AUC) quantification of the organoids at t = 60 min for stimulation with 0.128 µM fsk alone or in combination with CFTR modulators. The p.Phe508del/p.Phe508del organoids treated with iva/teza/elexa were used as a reference for maximal CFTR rescue. Dashed green and red lines represent the established thresholds for high and medium potential clinical benefit for treatments, respectively. Data represent the mean ± SEM of 6–10 replicates per condition. Organoid genotypes are indicated above panels. Significance was considered for *p*-value ≤ 0.05 (ANOVA) and symbols represents significance vs.: *—stimulation with fsk alone; #—stimulation with iva + fsk; \$—stimulation with teza/iva + fsk.

CFTR) after cloning of p.Arg334Trp-CFTR cDNA into lentiviral plasmids followed by site-directed mutagenesis as previously described (Canato et al., 2018; Awatade et al., 2019). CFBE cells expressing p.Phe508del-CFTR were a kind gift of Dr. Zsuzsa Bebok, United States (Bebok et al., 2005).

2.5 Western blot (WB) analysis

For CFTR protein detection, CFBE cells expressing p.Phe508del- or p.Arg334Trp-CFTR, respectively, were treated with 5 µM teza alone or combined with 3 µM elexa for 24 h, before being lysed and

analysed by WB as described (Ramalho et al., 2021). Images were acquired using ChemiDoc XRS+ (BioRad, United States) imaging system and the intensity of the bands was quantified by Image lab 4.0 software (BioRad, United States).

2.6 Ussing chamber recordings

CFBE cells expressing p.Arg334Trp-CFTR were seeded on collagen IV (Sigma-Aldrich) -coated Snapwell filter inserts (Corning, United States) and treated as above with CFTR modulator drugs. CFTR function was assessed in Ussing

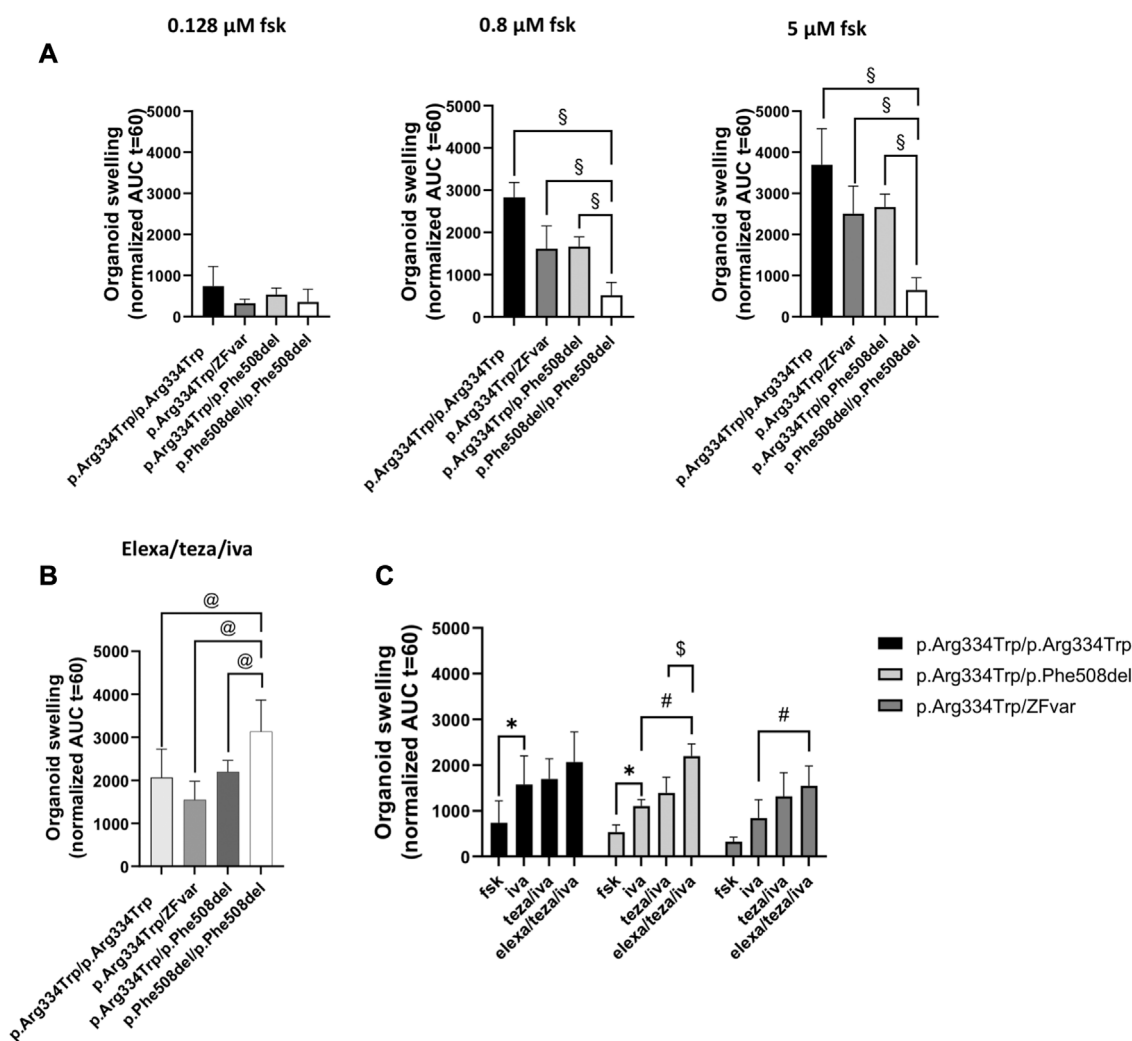


FIGURE 2

Comparison of the rescue of CFTR function by the three different modulators tested in intestinal organoids with p.Arg334Trp/p.Arg334Trp, p.Arg334Trp/ZFvar, and p.Arg334Trp/p.Phe508del genotypes. Organoid swelling is represented as area under the curve (AUC) of organoid surface area increase (baseline = 100%, t = 60 min) for stimulation with fsk alone or in combination with CFTR modulators. (A) Mean AUC of organoids carrying p.Arg334Trp/p.Arg334Trp, p.Arg334Trp/ZFvar, p.Arg334Trp/p.Phe508del at 0.128, 0.8 and 5 μM fsk alone (B) Mean AUC of organoids at 0.128 μM fsk treated with elexa/teza/iva. (C) Mean AUC of organoids carrying p.Arg334Trp/p.Arg334Trp, p.Arg334Trp/ZFvar and p.Arg334Trp/p.Phe508del at 0.128 μM Fsk treated with elexa/teza/iva. Data represent the mean \pm SEM of 6–10 replicates per condition. Significance was considered for p -value ≤ 0.05 and symbols represents significance vs.: §—stimulation with fsk alone in p.Phe508del/p.Phe508del organoids (ANOVA); @—stimulation with elexa/teza/iva in p.Phe508del/p.Phe508del organoids (ANOVA); *—stimulation with fsk alone (ANOVA); #—stimulation with iva + fsk (ANOVA); §—stimulation with teza/iva + fsk (ANOVA).

Chamber as previously described (Awatade et al., 2019). The transepithelial voltage (Vte) and transepithelial resistance (Rte) were recorded and used to calculate the equivalent short-circuit current (Isc-eq) using Ohm's law: $Isc = Vte/Rte$. Fsk was added apically followed by iva to cells pre-treated with DMSO or teza or elexa/teza for 24 h.

2.7 Statistical analyses

Statistical analyses were performed on GraphPad Prism 9.0 using ANOVA followed by a Dunnett's test (*post hoc* test), with p -value ≤ 0.05 considered as significantly different.

3 Results

3.1 Assessment of p.Arg334Trp-CFTR function in intestinal organoids and the effect of CFTR modulators

To assess the response of the p.Arg334Trp CFTR variant to modulators we first used the forskolin-induced swelling (FIS) assay in pwCF-derived organoids as previously described (Vonk et al., 2020). The assay uses forskolin (fsk), a CFTR agonist, to induce organoid swelling. Fsk promotes the opening of the CFTR channel leading to ion and water uptake and consequent organoid swelling. In the absence of functional CFTR, there is no swelling, and the

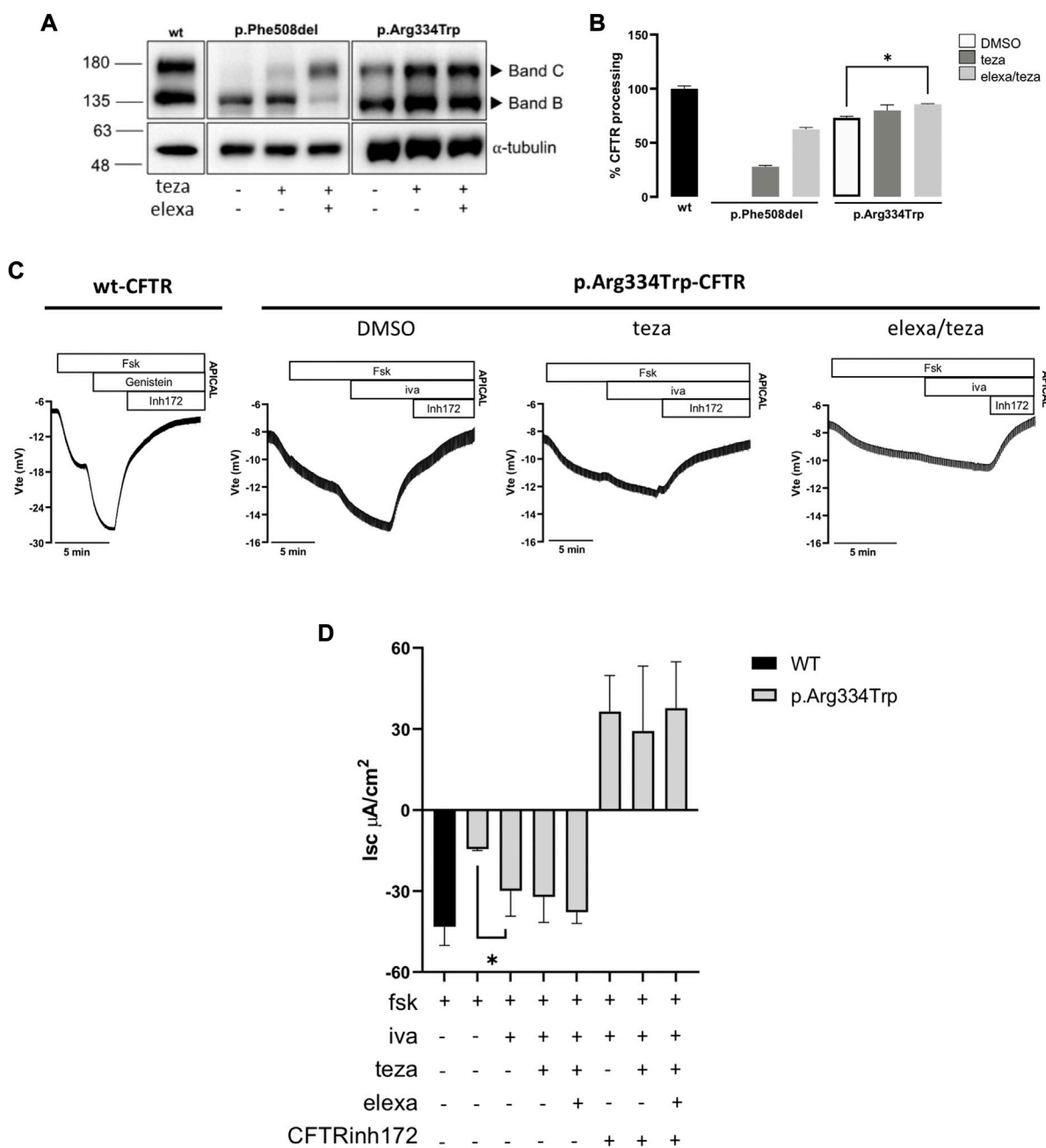


FIGURE 3 CFTR modulators effect on CFTR expression and function in CFBE cells expressing p.Arg334Trp-CFTR. **(A)** Western blot (WB) analysis of p.Arg334Trp-CFTR stably expressed in CFBE cells after 24 h treatment with 5 μM teza alone or combined with 3 μM elexa. Cells expressing F508del-CFTR were analysed as a control. As a loading control α-tubulin was used. **(B)** CFTR processing analysed as the percentage of band C to total CFTR and normalized to the efficiency of processing of wt-CFTR (shown as mean ± SEM of three replicates). Significance was considered for *p*-value ≤0.05 and * symbol represents significance vs. DMSO (ANOVA). **(C)** Original Ussing Chamber (short-circuit) recordings of CFTR-mediated chloride secretion measured as transepithelial voltage (*V_{te}*). Fsk was added apically followed by iva to cells pre-treated with DMSO or 5 μM teza alone or combined with 3 μM elexa. DMSO was used as vehicle control. **(D)** Summary of equivalent short-circuit currents (*I_{sc-eq}*) induced by fsk, iva + fsk, teza/iva + fsk and elexa/teza/iva + fsk. Values are represented as mean ± SEM (*n* = 4). Significance was considered for *p*-value ≤0.05 and * symbol represents significance vs. stimulation with fsk alone after DMSO (ANOVA).

organoid keeps the same size. This assay has been validated as a robust *ex vivo* biomarker and as a good predictor of clinical benefit for CFTR modulators (Boj et al., 2017). Organoids were pre-incubated with the corrector teza alone or combined with elexa for 24 h and then, right before starting the FIS assay, they were

acutely stimulated with fsk and iva. In total, we analysed rectal organoids from 10 individuals bearing the p.Arg334Trp variant (see Methods)—one homozygous, four heterozygous p.Arg334Trp/p.Phe508del, and five heterozygous for p.Arg334Trp with a zero-function variant (ZFvar) other than p.Phe508del (some authors call

these “minimal function” variants, but this designation is misleading as it may be wrongly interpreted as “residual function”). In parallel, organoids from an individual homozygous for p.Phe508del were used as a positive control for response to the modulator drugs.

Incubations with fsk alone show that organoids, either homozygous or heterozygous for the p.Arg334Trp variant, have residual CFTR function at fsk concentrations of 0.8 μ M and higher when compared with p.Phe508del homozygous organoids (Figures 1A–J, left panel, black line vs. grey dotted line with full dots; Figure 2A, middle and right panels).

For the p.Arg334Trp-homozygous organoids, stimulation with potentiator iva alone resulted in significant organoid swelling when compared with the basal conditions (Figure 1A, right panel, orange vs. black bar). In these organoids, pre-treatment with teza or elixa/teza did not induced further significantly organoid swelling when compared to treatment with iva alone.

In organoids from two out of four pwCF with genotype p.Arg334Trp/p.Phe508del, treatment with potentiator iva alone at the 0.128 μ M fsk concentration induced significant organoid swelling vs basal condition (Figures 1B, E, right panels, orange vs. black bar), but iva treatment was non-significant vs. baseline for the other two individuals (Figures 1C, D, right panels, orange vs. black bars). For organoids from these four pwCF, pre-treatment with teza only caused significant further swelling in organoids from one individual (Figure 1B, right panel, turquoise vs. orange bars), but not in those from the other 3 (Figures 1C–E, right panels, turquoise vs. orange bars). Nonetheless, a significant increase in swelling was always observed for organoids from these four individuals when stimulated with iva after pre-treatment with elixa/teza (Figures 1B–E, right panels, purple vs. orange bars).

Results for organoids from the five pwCF heterozygous for p.Arg334Trp and a non-p.Phe508del ZFvar, swelling after treatment with iva alone was only observed for organoids with the p.Arg1062X and p.Thr1086Pro variants (Figures 1F, J, right panels, orange vs. black bars, respectively). For these five organoids, pre-retreatment with teza only caused further significant swelling (vs. iva alone) when the second variant was p.Arg1162X, p.Gly542X, or p.Tyr1092X (Figures 1F–H, right panel, turquoise vs. orange bars), but not when it was p.Gln1100Pro, or p.Thr1086Pro (Figures 1I, J, right panels, turquoise vs. orange bars). However, for p.Arg334Trp/p.Gln1100Pro organoids teza/iva treatment caused significant swelling vs. fsk alone (Figure 1I, right panel, turquoise vs. black bars). The response of these organoids to elixa/teza/iva was similar to that observed for p.Arg334Trp/p.Phe508del organoids, i.e., in all five organoids significant swelling was observed vs. iva alone (Figures 1F–H, J, right panels, purple vs. orange bars) or vs. fsk (Figure 1I, right panel purple vs. black bars). However, the swelling observed for p.Arg334Trp/p.Phe508del and p.Arg334Trp/p.Arg334Trp organoids was higher by ~30%, and by ~25%, respectively from the swelling of organoids bearing p.Arg334Trp combined with non-p.Phe508del ZFvar, albeit these differences are not significant (Figure 2B). For organoids homozygous for p.Arg334Trp, treatment with potentiator iva was enough to rescue CFTR function, with no additional effect from the pre-treatment with correctors (teza and elixa) (Figure 2C, black bars). CFTR function was also rescued by iva alone in organoids heterozygous for p.Arg334Trp and p.Phe508del, however, elixa/teza/iva treatment resulted in further

significant increase in organoid swelling (Figure 2C, light grey bars). For organoids heterozygous for p.Arg334Trp and a non-p.Phe508del ZFvar a significant increase in organoid swelling was only observed after treatment with elixa/teza/iva (Figure 2C, dark grey bars).

3.2 Impact of p.Arg334Trp on CFTR processing/traffic in cell lines and effect of correctors

To characterize the effect of p.Arg334Trp on CFTR traffic, function, and response to CFTR modulators, we produced a human bronchial epithelial (CFBE) cell line expressing this variant individually (see Methods). Cells were incubated for 24 h with 5 μ M teza alone or combined with 3 μ M elixa or treated only with DMSO 0.1% (v/v), as the vehicle control. In parallel, these compounds were also tested on p.Phe508del-CFTR expressing CFBE cells as a control. Total protein was collected, and WB was used to assess the maturation status as a proxy for PM traffic of p.Arg334Trp-CFTR. Results show that both mature and immature forms of p.Arg334Trp-CFTR were detected under control conditions (Figures 3A, B). In fact, p.Arg334Trp-CFTR processing efficiency (measured by the ratio of band C to total CFTR) was 73% of wt-CFTR. No significant effect of pre-treatment with teza alone was observed, while when teza was combined with elixa, a small albeit significant increase in p.Arg334Trp-CFTR processing (13% vs. DMSO) was observed.

3.3 Assessment of p.Arg334Trp-CFTR function in cell lines and effect of correctors

To evaluate the function of p.Arg334Trp-CFTR and its response to CFTR modulators, we assessed transepithelial ion transport in micro-Ussing chamber of CFBE cells stably expressing this variant. Residual CFTR-mediated Cl^- secretion was elicited by fsk addition, indicating that p.Arg334Trp-CFTR has indeed residual function which corresponds to ~34% of wt-CFTR (Figures 3C, D). Similar to what we showed for the p.Arg334Trp-homozygous organoids, potentiation with iva alone was enough to rescue CFTR function. In fact, addition of iva alone further significantly increased the basal function by ~36% vs. DMSO alone and to ~69% of wt-CFTR (Figure 3D). Furthermore, consistently with the FIS data for the p.Arg334Trp-homozygous organoids, pre-treatment with correctors teza or elixa/teza did not further increase function of p.Arg334Trp-CFTR relative to iva alone (Figure 1A, right panel; Figure 3D).

4 Discussion

The rare p.Arg334Trp CFTR variant is described as CF-causing (CFTR, 2023) and it is not included in the list of variants eligible for treatment with the currently available CFTR modulators. In this study, we investigated the effect of CFTR modulators on p.Arg334Trp-CFTR in intestinal organoids derived from 10 pwCF having this mutation in homozygosity (1 individual) or in compound heterozygosity with p.Phe508del (4 individuals) or

with a ZFvar: p.Arg1162X, p.Gly542X, p.Tyr1092X, p.Gln1100Pro, p.Thr1086Pro (1 individual each). Moreover, we characterized the p.Arg334Trp variant for CFTR protein traffic/processing and function when expressed alone in the CFBE cell line.

The p.Arg334Trp-CFTR is a class IV rare variant characterized by decreased CFTR conductance. In the CFTR2 database, there are 408 individuals registered who carry this variant. Clinically, p.Arg334Trp-CFTR is associated with atypical forms of CF, and individuals bearing this variant are likely to be pancreatic sufficient (CFTR, 2023).

To investigate CFTR function in intestinal organoids, we performed the FIS assay, which is fully CFTR-dependent (Boj et al., 2017). We observed that intestinal organoids either homozygous or heterozygous for p.Arg334Trp-CFTR show residual function at fsk concentrations of 0.8 μ M and higher. These findings are in agreement with the results in the Ussing chamber for the p.Arg334Trp-CFTR variant stably expressed in CFBE cells which evidenced residual function (34% of wt-CFTR), which is consistent with defective conductance or lowered P_o (Sheppard et al., 1993; De Boeck and Amaral, 2016; Han et al., 2018; Veit et al., 2020). Intestinal organoids carrying p.Arg334Trp/p.Phe508del variants showed lower residual CFTR function at higher concentrations of fsk stimulation when compared with p.Arg334Trp/p.Arg334Trp organoids. As p.Phe508del is a ZFvar, the observed residual function is most likely due to the presence of p.Arg334Trp.

Ivacaftor alone was able to significantly potentiate this residual CFTR function in p.Arg334Trp/p.Arg334Trp organoids and in 4/9 of heterozygous organoids for p.Arg334Trp. Our data show that organoids with the same p.Arg334Trp/p.Phe508del genotype evidenced variable responses to CFTR modulators (2 positive responses in four organoids), thus highlighting individual-specific differences in response to CFTR modulators. In the Ussing chamber, CFBE expressing p.Arg334Trp-CFTR also responded to iva stimulation by showing a further increase in function of 36% (reaching 69% of wt-CFTR). These data are also consistent with the FIS results in p.Arg334Trp-homozygous organoids that evidenced a 53% increase vs. basal swelling. It was previously shown that p.Arg334Trp-CFTR, when expressed in Fischer rat thyroid (FRT) cells, is normally processed and exhibits very low CFTR channel activity (Van Goor et al., 2014; Phuan et al., 2019), but with no further potentiation by iva. However, in CFBE cells, which are from human airway origin, a small but significant (Veit et al., 2020) increase has been observed upon iva treatment which is in line with our results. This discrepancy between FRT cells on the one hand and human bronchial epithelial cell line and pwCF-derived organoids on the other, highlight the superiority of the latter to predict responses to CFTR modulator drugs.

Western blot analysis for the CFBE cell line stably expressing p.Arg334Trp-CFTR detected both immature and mature forms of p.Arg334Trp-CFTR under control conditions, with a processing of 73% of wt-CFTR. Results also show that p.Arg334Trp-CFTR processing significantly increased by 13% after correction with the teza/elexa combination, but not with teza alone. Data from both FIS, in p.Arg334Trp-homozygous organoids, and Ussing chamber in CFBE cell expressing p.Arg334Trp-CFTR, show that pre-treatment with either teza or elexa followed by stimulation with iva did not increase CFTR function when compared to iva treatment alone. This may appear as a discrepancy regarding the

data on processing obtained by WB. However, it should be emphasized that cells analysed by WB were not subjected to iva. In fact, this compound has been shown to decrease the efficiency of correctors, which may explain the absence of an additional functional response (Cholon et al., 2014; Veit et al., 2014).

FIS data in organoids from 8/9 individuals heterozygous for p.Arg334Trp showed that the triple combination (elexa/teza/iva) induced a higher level of organoid swelling when compared to iva alone, suggesting additional rescue of CFTR by the elexa/teza combination. However, four of these eight organoids had p.Phe508del on the second allele, a variant known to respond to elexa/teza/iva. Indeed, the triple combination of elexa/teza/iva is approved for the treatment of individuals carrying only one copy of the p.Phe508del variant. It is important to mention that the effect of the triple combination in p.Arg334Trp/p.Phe508del was statistically lower, in all four subjects, when compared with the control p.Phe508del/p.Phe508del. These results suggest a low impact of the correctors teza and elexa on the rescue of p.Arg334Trp-CFTR.

Among the other four organoids which responded more to elexa/teza/iva than to iva alone were those with p.Arg334Trp-CFTR in combination with p.Thr1086Pro or with a PTC (premature termination codon) variant, namely: p.Gly542X, p.Tyr1092X and p.Arg1162X. In fact, these three organoids also responded significantly more to teza/iva than to iva alone. As PTC variants are not likely to respond to modulators, the fact that response was only observed in the presence of correctors may suggest the need to increase maturation of p.Arg334Trp-CFTR for the potentiation effect of iva to be observed. In fact, we confirmed this increase in processing of p.Arg334Trp-CFTR by correctors in the cell line. Interestingly, the p.Arg334Trp/p.Arg1162X organoids showed a higher rescue of CFTR function, 48% and 38% more to elexa/teza/iva when compared with p.Arg334Trp/p.Gly542X and p.Arg334Trp/p.Tyr1092X, respectively. As the p.Arg1162X variant is closer to CFTR C-terminus, it could result in the production of small amounts of truncated CFTR partly rescuable by the modulators, as has been suggested for other non-sense variants such as W1282X (Haggie et al., 2017). Notwithstanding, these results may also result from a potential impact of modifier genes which contribute to the variation of CFTR rescue of those phenotypes (O'Neal and Knowles, 2018).

CFTR function rescue by CFTR modulators was also observed in intestinal organoids from individuals heterozygous for p.Arg334Trp-CFTR and p.Gln1100Pro or p.Thr1086Pro, respectively. The variant p.Gln1100Pro is very rare and characterized by defective CFTR protein folding (class II), which was shown to be rescued by the double combination of elexa/teza (Ramalho et al., 2021). The p.Thr1086Pro, is a missense variant and is not described in CFTR2 database. Both individuals carrying these variants are not eligible for the currently available CFTR modulator therapies. However, we show here that individuals having these variants combined with p.Arg334Trp are likely to have clinical benefit from CFTR modulator treatments, as the respective purple bars (Figures 1I,J, right panels) are above the dotted red line representing the established threshold for medium potential clinical benefit and very close (p.Arg334Trp/p.Gln1100Pro) or above (p.Arg334Trp/p.Thr1086Pro) the threshold for high potential clinical benefit (green dotted line). However, we do not know whether these variants in genotypes with other ZFvar will respond to CFTR modulators.

Taken together, our results provide evidence that p.Arg334Trp can be rescued by current CFTR modulators when in homo- or heterozygosity with a ZFvar. We can thus predict that individuals with this variant in at least one of their CFTR alleles will likely benefit from current CFTR modulator drugs (at least one orange, turquoise or purple bar in each right panel of (Figure 1), is above the red line and very close or above the green dotted line, representing the established thresholds for high and medium potential clinical benefit for treatments, respectively).

Although responses to teza/iva or to elixa/teza/iva in organoids with p.Arg334Trp in homozygosity or combined with p.Phe508del were usually higher than those in organoids with p.Arg334Trp combined with a non-p.Phe508del ZFvar, the differences were not found to be significant (Figure 2B). Moreover, as different responses were observed for the same genotype (p.Arg334Trp/p.Phe508del), as well as for a group of variants in the same class (p.Gly542X, p.Tyr1092X and p.Arg1162X), our data evidence the differences among individuals with the same genotype, thus highlighting the importance of personalized medicine in CF, which is of special relevance for pwCF carrying rare variants and thus unlikely to enter “classical” clinical trials.

Finally, as individuals with very rare variants are not eligible to participate in ‘classical’ clinical trials due to their low numbers (and geographic dispersion), we recommend that the precision/personalized approach adopted here should be taken into account for drug reimbursement policies by health insurance systems/national health services.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Hospital de Santa Maria, Lisboa, Portugal (DIRCLN-16JUL 2014–211). The patients/participants provided their written informed consent to participate in this study.

References

- Awatade, N. T., Ramalho, S., Silva, I. A. L., Felicio, V., Botelho, H. M., de Poel, E., et al. (2019). R560S: A class II CFTR mutation that is not rescued by current modulators. *J. Cyst. Fibros.* 18 (2), 182–189. doi:10.1016/j.jcf.2018.07.001
- Awatade, N. T., Uliyakina, I., Farinha, C. M., Clarke, L. A., Mendes, K., Sole, A., et al. (2015). Measurements of functional responses in human primary lung cells as a basis for personalized therapy for cystic fibrosis. *EBioMedicine* 2 (2), 147–153. doi:10.1016/j.ebiom.2014.12.005
- Bebok, Z., Collawn, J. F., Wakefield, J., Parker, W., Li, Y., Varga, K., et al. (2005). Failure of cAMP agonists to activate rescued deltaF508 CFTR in CFBE41o-airway epithelial monolayers. *J. Physiol.* 569 (2), 601–615. doi:10.1113/jphysiol.2005.096669
- Boj, S. F., Vonk, A. M., Statia, M., Su, J., Vries, R. R. G., Beekman, J. M., et al. (2017). Forskolin-induced swelling in intestinal organoids: An *in vitro* assay for assessing drug response in cystic fibrosis patients. *JoVE* 120, 55159. doi:10.3791/55159
- Canato, S., Santos, J. D., Carvalho, A. S., Aloria, K., Amaral, M. D., Matthiesen, R., et al. (2018). Proteomic interaction profiling reveals KIFC1 as a factor involved in early targeting of F508del-CFTR to degradation. *Cell Mol. Life Sci.* 75 (24), 4495–4509. doi:10.1007/s00018-018-2896-7
- Cfmdb (2021). Cystic fibrosis mutation database. <http://www.genet.sickkids.on.ca/StatisticsPage.html> (Accessed April 12).
- Cfmdb (2023). Cystic fibrosis mutation database. <http://www.genet.sickkids.on.ca/> (Accessed January 16).
- Cftr (2023). CFTR2 database. <https://cftr2.org/mutation/general/R334W/> (Accessed January 16).
- Cholon, D. M., Quinney, N. L., Fulcher, M. L., Esther, C. R., Das, J., Dokholyan, N. V., et al. (2014). Potentiator ivacaftor abrogates pharmacological correction of Δ F508 CFTR in cystic fibrosis. *Sci. Transl. Med.* 6 (246), 246ra96. doi:10.1126/scitranslmed.3008680
- Cutting, G. R. (2015). Cystic fibrosis genetics: From molecular understanding to clinical application. *Nat. Rev. Genet.* 16 (1), 45–56. doi:10.1038/nrg3849
- Dalemans, W., Barbry, P., Champigny, G., Jallat, S., Dott, K., Dreyer, D., et al. (1991). Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. *Nature* 354 (6354), 526–528. doi:10.1038/354526a0
- De Boeck, K., and Amaral, M. D. (2016). Progress in therapies for cystic fibrosis. *Lancet Respir. Med.* 4 (8), 662–674. doi:10.1016/S2213-2600(16)00023-0

Author contributions

VR and CSR: conceptualization, formal analysis, validation, investigation, visualization, methodology and writing-original draft SSR: formal analysis and methodology IS: formal analysis and methodology JB: methodology CMF: supervision, funding acquisition, investigation, writing-review and editing IP: conceptualization, and writing-review and editing MDA: resources, supervision, funding acquisition, investigation, project administration, and writing-review and editing.

Funding

Work supported by UIDB/04046/2020 and UIDP/04046/2020 centre grants (to BioISI), from FCT/MCTES Portugal, and research grants “HIT-CF” (H2020-SC1-2017–755021) from EU; SRC 013 from CF Trust-UK (to MDA) and FARINH1910 from Cystic Fibrosis Foundation (to CMF). SSR, CSR and VR are recipients of PhD fellowships SFRH/BD/142857/2018 and SFRH/BD/153053/2022 from BioSys PhD programme PD/00065/2012, SFRH/BD/06718/2021, respectively all from FCT (Portugal).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

- Ecfs (2020). ECFS patient registry. <https://www.ecfs.eu/projects/ecfs-patient-registry/annual-reports>.
- Guo, J., Garratt, A., and Hill, A. (2022). Worldwide rates of diagnosis and effective treatment for cystic fibrosis. *J. Cyst. Fibros.* 21 (3), 456–462. doi:10.1016/j.jcf.2022.01.009
- Hagemeyer, M. C., Vonk, A. M., Awatade, N. T., Silva, I. A. L., Tischer, C., Hilsenstein, V., et al. (2021). An open-source high-content analysis workflow for CFTR function measurements using the forskolin-induced swelling assay. *Xu J. Ed. Bioinforma.* 36 (24), 5686–5694. doi:10.1093/bioinformatics/btaa1073
- Haggie, P. M., Phuan, P. W., Tan, J. A., Xu, H., Avramescu, R. G., Perdomo, D., et al. (2017). Correctors and potentiators rescue function of the truncated W1282x-cystic fibrosis transmembrane regulator (CFTR) translation product. *J. Biol. Chem.* 292 (3), 771–785. doi:10.1074/jbc.M116.764720
- Han, S. T., Rab, A., Pellicore, M. J., Davis, E. F., McCague, A. F., Evans, T. A., et al. (2018). Residual function of cystic fibrosis mutants predicts response to small molecule CFTR modulators. *JCI Insight* 3 (14), e121159. doi:10.1172/jci.insight.121159
- Jih, K. Y., and Hwang, T. C. (2013). Vx-770 potentiates CFTR function by promoting decoupling between the gating cycle and ATP hydrolysis cycle. *Proc. Natl. Acad. Sci. U. S. A.* 110 (11), 4404–4409. doi:10.1073/pnas.1215982110
- Middleton, P. G., Mall, M. A., Dřevinec, P., Lands, L. C., McKone, E. F., Polineni, D., et al. (2019). Elexacaftor–Tezacaftor–Ivacaftor for Cystic Fibrosis with a Single Phe508del Allele. *N. Engl. J. Med.* 381 (19), 1809–1819. doi:10.1056/NEJMoa1908639
- O’Neal, W. K., and Knowles, M. R. (2018). Cystic fibrosis disease modifiers: Complex genetics defines the phenotypic diversity in a monogenic disease. *Annu. Rev. Genomics Hum. Genet.* 19, 201–222. doi:10.1146/annurev-genom-083117-021329
- Phuan, P. W., Tan, J. A., Rivera, A. A., Zlock, L., Nielson, D. W., Finkbeiner, W. E., et al. (2019). Nanomolar-potency “co-potentiator” therapy for cystic fibrosis caused by a defined subset of minimal function CFTR mutants. *Sci. Rep.* 9 (1), 17640. doi:10.1038/s41598-019-54158-2
- Ramalho, S. S., Silva, I. A. L., Amaral, M. D., and Farinha, C. M. (2021). Rare trafficking CFTR mutations involve distinct cellular retention machineries and require different rescuing strategies. *IJMS* 23 (1), 24. doi:10.3390/ijms23010024
- Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., GrZelcZak, Z., et al. (1989). Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science* 245 (4922), 1066–1073. doi:10.1126/science.2475911
- Rowe, S. M., Miller, S., and Sorscher, E. J. (2005). Cystic fibrosis. *N. Engl. J. Med.* 352 (19), 1992–2001. doi:10.1056/NEJMra043184
- Sabusap, C. M., Wang, W., McNicholas, C. M., Chung, W. J., Fu, L., Wen, H., et al. (2016). Analysis of cystic fibrosis-associated P67L CFTR illustrates barriers to personalized therapeutics for orphan diseases. *JCI Insight* 1 (14), e86581. doi:10.1172/jci.insight.86581
- Sheppard, D. N., Rich, D. P., Ostedgaard, L. S., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1993). Mutations in CFTR associated with mild-disease-form Cl-channels with altered pore properties. *Nature* 362 (6416), 160–164. doi:10.1038/362160a0
- Sheppard, D. N., and Welsh, M. J. (1999). Structure and function of the CFTR chloride channel. *Physiol. Rev.* 79 (1), S23–S45. doi:10.1152/physrev.1999.79.1.S23
- Taylor-Cousar, J. L., Munck, A., McKone, E. F., van der Ent, C. K., Moeller, A., Simard, C., et al. (2017). Tezacaftor–Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del. *N. Engl. J. Med.* 377 (21), 2013–2023. doi:10.1056/NEJMoa1709846
- Van Goor, F., Straley, K. S., Cao, D., Gonzalez, J., Hadida, S., Hazlewood, A., et al. (2006). Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. *Am. J. Physiol. Lung Cell Mol. Physiol.* 290 (6), L1117–L1130. doi:10.1152/ajplung.00169.2005
- Van Goor, F., Yu, H., Burton, B., and Hoffman, B. J. (2014). Effect of ivacaftor on CFTR forms with missense mutations associated with defects in protein processing or function. *J. Cyst. Fibros.* 13 (1), 29–36. doi:10.1016/j.jcf.2013.06.008
- Veit, G., Avramescu, R. G., Perdomo, D., Phuan, P. W., Bagdany, M., Apaja, P. M., et al. (2014). Some gating potentiators, including VX-770, diminish Δ F508-CFTR functional expression. *Sci. Transl. Med.* 6 (246), 246ra97. doi:10.1126/scitranslmed.3008889
- Veit, G., Da Fonte, D. F., Avramescu, R. G., Premchandrar, A., Bagdany, M., Xu, H., et al. (2020). Mutation-specific dual potentiators maximize rescue of CFTR gating mutants. *J. Cyst. Fibros.* 19 (2), 236–244. doi:10.1016/j.jcf.2019.10.011
- Vonk, A. M., van Mourik, P., Ramalho, A. S., Silva, I. A. L., Statia, M., Kruisselbrink, E., et al. (2020). Protocol for application, standardization and validation of the forskolin-induced swelling assay in cystic fibrosis human colon organoids. *Star. Protoc.* 1 (1), 100019. doi:10.1016/j.xpro.2020.100019
- Wainwright, C. E., Elborn, J. S., Ramsey, B. W., Marigowda, G., Huang, X., Cipolli, M., et al. (2015). Lumacaftor–Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N. Engl. J. Med.* 373 (3), 220–231. doi:10.1056/NEJMoa1409547