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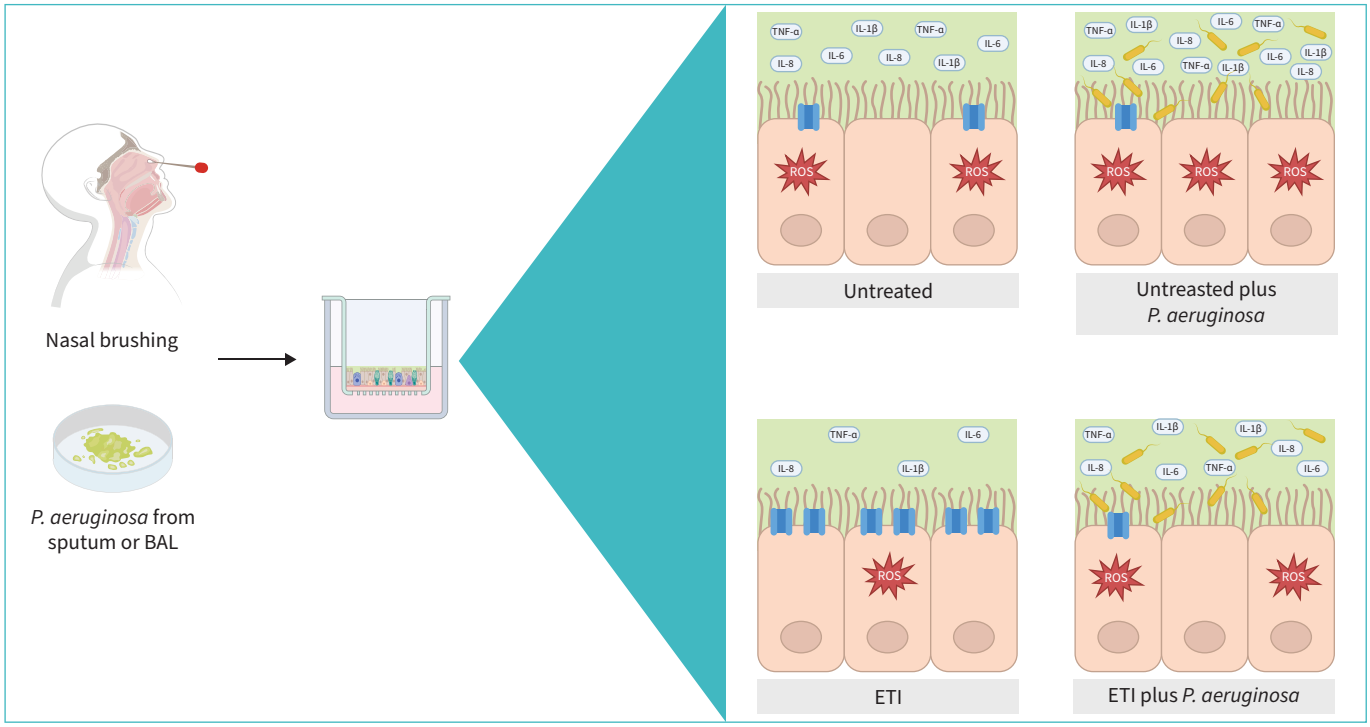

GRAPHICAL ABSTRACT *Pseudomonas aeruginosa* infection reduces ETI treatment efficacy. BAL: bronchoalveolar lavage; ETI: elexacaftor/tezacaftor/ivacaftor.

Deleterious effect of *Pseudomonas aeruginosa* on F508del-CFTR rescued by elexacaftor/tezacaftor/ivacaftor is clinical strain-dependent in patient-derived nasal cells

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Shareable abstract (@ERSpublications)
Under *in vitro* conditions, *P. aeruginosa* infection impacts the efficacy of ETI treatment, likely representing a considerable contributor to the variability of clinical responses observed in *in vivo* studies https://bit.ly/48Q1y7O

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Abstract

Background The triple cystic fibrosis transmembrane conductance regulator (CFTR) modulators combination elexacaftor/tezacaftor/ivacaftor (ETI) has been approved for people with cystic fibrosis (pwCF) bearing at least one *F508del* allele. Despite the development of CFTR modulators having dramatically improved respiratory outcomes in pwCF, clinical studies have showed variable responses to this drug formulation. Of note, airway inflammation and bacterial colonisation persist in the upper and lower respiratory tract even in ETI-treated patients.

Methods We first tested the clinical exoproducts (EXO) of *Pseudomonas aeruginosa* isolated from 15 CF patients in wild-type (WT) and F508del-CFTR CF bronchial epithelial (CFBE) cells. We were then prompted to evaluate the effects of EXO in *ex-vivo* patient-derived tissues. Therefore, we cultured primary nasal epithelial cells (HNECs) with EXO isolated from the corresponding pwCF to mimic the native status of CF airway.

Results We found that EXO variably decreased WT-, F508del- and ETI-dependent F508del-CFTR function and increased proinflammatory cytokines and reactive oxygen species (ROS) levels in a clinical strain-specific manner. Similarly, we observed a variable reduction of F508del-CFTR function in presence or absence of ETI and upregulation of proinflammatory cytokines and ROS levels. Interestingly, HNECs treated with EXO isolated from the corresponding donor and three different pwCF showed a variable reduction of ETI-dependent F508del-CFTR function mainly due to clinical strains with limited effect of patient background. Furthermore, we demonstrated that ETI pretreatment decreased the cytokines and ROS levels down to the levels of uninfected cells.

Conclusion These preclinical studies suggest that *in vitro* screening of patient-specific response to CFTR modulators under infection/inflammation conditions could prove to be a valuable tool to enhance the prediction of clinical response.

Introduction

Cystic fibrosis (CF) is an autosomal recessive, multiorgan, lethal disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The prevalent disease-causing mutation, the

deletion of Phenylalanine at 508 position (F508del), affects ∼90% of people with CF (pwCF), with marked regional differences.

pwCF are prone to contracting bacterial lung infections caused by the opportunistic pathogen *Pseudomonas aeruginosa* [1]. There has been significant progress in the development of modulators that target the molecular and functional defects caused by the F508del mutation: triple combination of CFTR modulators (elexacaftor–tezacaftor–ivacaftor (ETI)) was recently approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2019 and 2020, respectively, for patients carrying at least one F508del allele. Phase 2 and 3 clinical trials confirmed substantial beneficial effects on the forced expiratory volume in 1 s (FEV_1), pulmonary exacerbations, sweat chloride concentration and body mass index [2, 3]. Despite the development of CFTR modulators has dramatically improved respiratory outcomes in pwCF, airway inflammation and bacterial colonisation persist even in pwCF treated with modulator therapies [4, 5]. On the other hand, ETI treatment is associated with reduced *P. aeruginosa* load in respiratory cultures, decreased circulating neutrophils and levels of proinflammatory cytokines after a year of therapy [6]. *P. aeruginosa* infections not only play a detrimental role in airway integrity, but also impact CFTR expression and function [7, 8]. In addition, it has been previously reported that isolated clinical *P. aeruginosa* or clinical exoproducts isolated from *P. aeruginosa* negatively impacted WT-CFTR and lumacaftor/ivacaftor-induced F508del-CFTR function and expression in both human CF bronchial epithelial (CFBE) cells and primary human airway epithelial cells isolated from non-CF and pwCF [9–11]. Nevertheless, the relationship between abnormal CFTR function and inflammation/infection has not been clearly defined. Despite ETI improving the quality of life in pwCF, clinical studies demonstrated a high patient-to-patient variation in ETI response that may feasibly be attributed in part to differences in microbial infections. We have already demonstrated that lipopolysaccharide (LPS) from *P. aeruginosa* reduces ETI-mediated rescue of F508del-CFTR in human bronchial epithelial cell line and primary nasal epithelial cells (HNECs) [12, 13]. Therefore, *in vitro* analyses (performed on primary airway epithelial cells) of patient-specific response to CFTR modulators, upon infection or inflammatory condition, could enhance the prediction of clinical response. In the current work, we aimed to evaluate the effect of clinical isolated exoproduct (EXO) of *P. aeruginosa*, obtained from the sputum of pwCF, on the inflammatory response, reactive oxygen species (ROS) levels and F508del-CFTR rescue by ETI both in CFBE41o- cells stably expressing WT- or F508del-CFTR (CFBE) and in HNECs.

Material and methods

Patients

Nasal epithelial cells were obtained from non-CF and CFTR modulator-naïve pwCF enrolled at Bambino Gesù Hospital (Rome, Italy) and Giannina Gaslini Hospital (Genoa, Italy). This study was approved by the Research Ethics Board of Bambino Gesù Hospital (2961/2022) and Giannina Gaslini Hospital (CER 28/2020). All study participants or their guardians signed an informed consent.

Cell culture

CFBE41o- cells stably expressing WT- or F508del-CFTR (CFBE) were originally obtained from Dr Gruenert (University of California, USA). The cells were maintained in minimum essential medium (MEM) (Corning, New York, USA) supplemented with 10% fetal bovine serum (FBS) (Corning), L-glutamine (Corning) and penicillin/streptomycin (Corning) at 37°C with 5% CO_2, as previously described [14]. A concentration of 0.5 $\mu g \cdot mL^{-1}$ puromycin (Sigma-Aldrich, Milan, Italy) was used as a positive selection for WT-CFTR and 2 $\mu g \cdot mL^{-1}$ puromycin for the selection of F508del-CFTR CFBE cells.

HNECs were isolated from brushing and subsequently cultured as previously described [14–17]. Briefly, HNECs were either seeded on collagen-coated 6.5-mm diameter transwell inserts (cat. #3470, Corning) or on 12-mm diameter transwell inserts (cat. #3801, Corning). Once confluent, the cells were cultured for 16–18 days at an air–liquid interface (ALI) with basal differentiation media (PneumaCult-ALI; STEMCELL Technologies, Canada).

P. aeruginosa strains isolation from the sputum

During patients' follow-up at our hospital, standard sputum culture was performed according to national guidelines for CF patient respiratory sample investigation (https://www.sifc.it/wp-content/uploads/2020/09/Raccomandazioni-Gruppo-Microbiologi-SIFC-2018.pdf). Colonies growing on agar plates, and considered putative pathogens, were subcultured on MacConkey agar and Columbia agar with 5% sheep blood (bioMérieux, Marcy-l'Étoile, France) and subsequently identified by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS; Bruker Daltonics, Bremen, Germany). Isolates identified as *P. aeruginosa* were then used for the following experiments.

Preparation of exoproducts of clinical P. aeruginosa *strains*

Exoproducts from clinical strains of *P. aeruginosa* (EXO) from the corresponding donor were obtained as previously described [9, 18]. In brief, *P. aeruginosa* strains were isolated from the sputum of a pwCF enrolled in this study and were grown in lysogeny broth (LB) medium for 72 h (corresponding to the late stationary phase) at 37°C with shaking at 250 rpm. The bacterial cultures supernatant was filtered successively through 0.8, 0.45 and 0.2 µm filters and aliquots of exoproducts were stored at −80°C [7]. LB was used as the control condition.

Protease activity assay

The activity of clinically isolated *P. aeruginosa*-secreted protease was measured by Hide-Remazol Brilliant Blue R (Sigma-Aldrich) as a substrate [18]. Briefly, 1 mL EXO was incubated with 15 mg hide azure blue reagent in 0.5 mL of 10 mM Tris (pH 7.5) for 1 h at 37°C with shaking at 250 rpm. To measure protease activity in EXO, the solution was centrifuged at 3000 g for 10 min and absorbance at 595 nm was quantified in the EXO by using a microplate reader (FilterMax F5, Molecular Devices).

Treatment of bronchial and nasal cells with clinical exoproducts of P. aeruginosa

CFBE cells were seeded on 96-well clear-bottom culture plates (Corning) and cultured at 37°C for 5 days post-confluence. Cells were treated with 0.1% DMSO, 3 µM VX-661+3 µM VX-445 (Selleck Chemicals) in the presence of vehicle (LB) or of 1 µM LPS from *P. aeruginosa* (Sigma-Aldrich) or of clinical exoproducts of *P. aeruginosa* (EXO) isolated from pwCF for 24 h.

HNECs were seeded on a 24-transwell (6.5 mm diameter, 0.4 µm pore size, Corning) or 96-transwell plate (4.26 mm diameter, 0.4 µm pore size, Corning) and cultured at 37°C for 16–18 days. Cells were treated at the basolateral region with 0.1% DMSO, 3 µM VX-661+3 µM VX-445 (Selleck Chemicals) and at the apical region with either 50 µL of vehicle (LB) for 24-transwell or 25 µL for 96-transwell plates or EXO isolated from the corresponding pwCF for 24 h [9, 18].

Fluorescence-based membrane polarisation assay

The cells were loaded with FLIPR blue membrane potential dye (cat #R8034; Molecular Devices) dissolved in chloride-free buffer [19]. The plate was read in a fluorescence plate reader (excitation 535 nm, emission 595 nm; FilterMax F5, Molecular Devices) at 37°C. CFTR function was measured after the acute addition of 1 µM FSK (for WT-CFTR, Sigma-Aldrich) or 10 µM FSK+ 1 µM VX-770 (for F508del-CFTR, Selleck Chemicals). Next, CFTR inhibitor (CFTRinh-172, 10 µM) was added to deactivate CFTR. The peak changes in fluorescence to CFTR agonist were normalised relative to the baseline fluorescence ($\Delta F/F_0$) [20, 21].

Ussing chamber studies

Short-circuit current (*Isc*) measurements were conducted in HNEC cultures with a Ussing chamber system using a voltage clamp (VCC MC6, Physiologic Instruments). The apical hemichamber was filled with 4 mL of sodium gluconate ringer (145 mM Na^+ gluconate, 3.3 mM K_2HPO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.4), and the basolateral hemichamber was filled with 4mL of NaCl ringer (145 NaCl, 3.3 mM K_2HPO_4, 1.2 $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.4). Alternatively for the experiments on HNEC carrying mutations not responsive to ETI, both hemichambers were filled with NaCl ringer (126 mM NaCl, 0.38 mM KH_2PO_4, 2.13 mM K_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 24 mM $NaHCO_3$ and 10 mM glucose) [17]. Both sides were continuously bubbled with a 5% CO_2–95% air mixture and the temperature of the solution was kept at 37°C. The transepithelial voltage was clamped at 0 mV after correcting voltages offset, and current and resistance were recorded at 37°C with the Acquire and Analyze package (Physiologic Instruments).

Cytokines secretion

Released interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α) were determined using ELISA kit (Antibodies, Stockholm, Sweden) in supernatants collected from HNEC cultures treated with 3 µM VX-661+3 µM VX-445+1 µM VX-770±EXO from the corresponding pwCF for 24 h at 37°C.

RNA extraction and quantification

Cells were lysed and RNA was extracted according to the manufacturer's protocol (Qiagen Micro Kit) as previously described [12]. cDNA was retrotranscribed from 1 µg of total RNA with iSCRIPT cDNA synthesis kit (Biorad, Hercules, USA). Quantitative real-time PCR was performed using EvaGreen (Ssofast EvaGreen, Biorad) fluorophore in 96-well plates and normalised to GADPH. The primer sets used for amplification were previously reported [13].

ROS measurements

Cells were incubated with 10 µM of ROS-sensitive dye o5-(and 6)-chloromethyl 2′, 7′-dichlorodihydrofluorescein diacetate acetyl ester (H_2DCFDA; Invitrogen, Waltham, USA) at 37°C for 30 min. ROS levels were quantified by measuring the fluorescence at 37°C by using a microplate reader (excitation: 485 nm, emission: 535 nm; FilterMax F5, Molecular Devices).

Statistical analysis

All data are represented as mean±SEM of at least three independent replicates. GraphPad 9.0 software (San Diego, CA, USA) was used for all statistical analysis. The paired two-tailed t-test or one-way/two-way ANOVA were conducted as appropriate with a significance level $p<0.05$. Data with multiple comparison were assessed using Tukey's multiple comparison test with $\alpha=0.05$.

Results

Impact of P. aeruginosa *exoproducts on proinflammatory cytokine expression and CFTR function in CF bronchial epithelial cells*

To understand the interplay between infection, inflammation and CFTR function, we first evaluated the impact of *P. aeruginosa* infection on airway epithelial cells. As expected, LPS treatment significantly increased the mRNA expression levels of proinflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) in both WT- and F508del-CFTR CFBE cells (supplementary figure S1A), downregulated by ETI treatment. We also observed that LPS reduced (around 10%) both WT-CFTR and ETI-mediated F508del-CFTR function in CFBE cells (supplementary figure S1B–C). We then generated EXO from clinical strains of *P. aeruginosa* isolated from the bronchoalveolar lavage (BAL) or sputum from 15 pwCF heterozygous for the *F508del* mutation (figure 1a), and to be sure that the effects induced on airway epithelial cells are not dependent on bacterial load, we evaluated the protease activity, which appeared to be similar across the clinical EXOs (optical density: mean±SD 0.9464±0.1022). We then exposed WT and F508del-CFTR CFBE cells to the different clinical EXOs. Figure 1b–c show the high variability of IL-1β, IL-6, IL-8 and TNF-α expression levels observed in WT and F508del-CFTR CFBE cells following treatment with the EXOs. The dashed line shows, as comparison, the mean cytokines mRNA levels, measured in CFBE cells treated with LPS, typically used to induce an inflammatory response (supplementary figure S1A). Furthermore, nine out of 15 EXOs reduced the WT-CFTR function by 12–67% in CFBE cells (figure 2a–b), whereas the F508del-CFTR activity appeared to be significantly decreased by 25–70% in the presence of four out of 15 clinical EXOs (figure 2c–d). However, ETI-mediated F508del-CFTR activity was significantly reduced by 11–53% in the presence of 10 out of 15 clinical EXOs in a strain-specific manner (figure 2d,e).

P. aeruginosa *exoproducts induce variable effects in nasal epithelial cells isolated from the corresponding donor*

We then aimed to investigate whether the different deleterious effects of EXO treatment on ETI-rescued F508del-CFTR function observed in CFBE cells occurred also in primary airway epithelial cells exposed to EXO obtained from the *P. aeruginosa* strain from the corresponding pwCF. Patient-derived cultures were generated from nasal brushing obtained from 15 individuals heterozygous for *F508del* mutation from whom the *P. aeruginosa* exoproducts were isolated. Clinical characteristics of the selected cohort, including CFTR genotype and microbiology, are summarised in figure 3a. Clinical data (figure 3a) on the FEV_1% and sweat chloride levels show that following 6 months of treatment with Kaftrio, the FEV_1% was significantly increased by 10.63 (figure 3a–b), while the sweat chloride levels were significantly reduced (mean −39.37). However, following 6 months of Kaftrio treatment, the lung clearance index (LCI) was not changed.

To investigate the effect of exposure to *P. aeruginosa* EXOs on CFTR function, we measured Cl^- conductance in HNECs by Ussing chamber studies (figure 4). These measurements were performed in well-differentiated HNEC cultures obtained from four non-CF donors as well as from the 15 subjects with CF from whom the *P. aeruginosa* exoproducts were isolated (figure 4a). Cells were treated with LB (vehicle) or EXO in the absence or presence of VX-445+VX-661 for 24 h before I_{sc} was recorded. In HNECs, 24 h incubation with the corresponding EXO reduced CFTR function by 3–50% (except for CF8 and CF9) (figure 4b–d). Furthermore, ETI treatment led to a patient-to-patient variable increase in CFTR function measured as ΔIsc $CFTR_{inh172}$ ($\mu A \cdot cm^{-2}$) (figure 4b-d), corresponding to 30–52% of CFTR function measured in HNECs from non-CF donors. However, in the presence of EXO, $CFTR_{inh172}$-sensitive CFTR current rescued by ETI was reduced by 14–64% (with the exception of CF8 and CF9), although CFTR function remained significantly higher than DMSO control (figure 4d). CFTR function (expressed as % of the WT value) significantly correlated with sweat chloride and % FEV_1 (supplementary figure S2). However, no significant correlation was observed between the ΔIsc $CFTR_{inh172}$ and LCI.

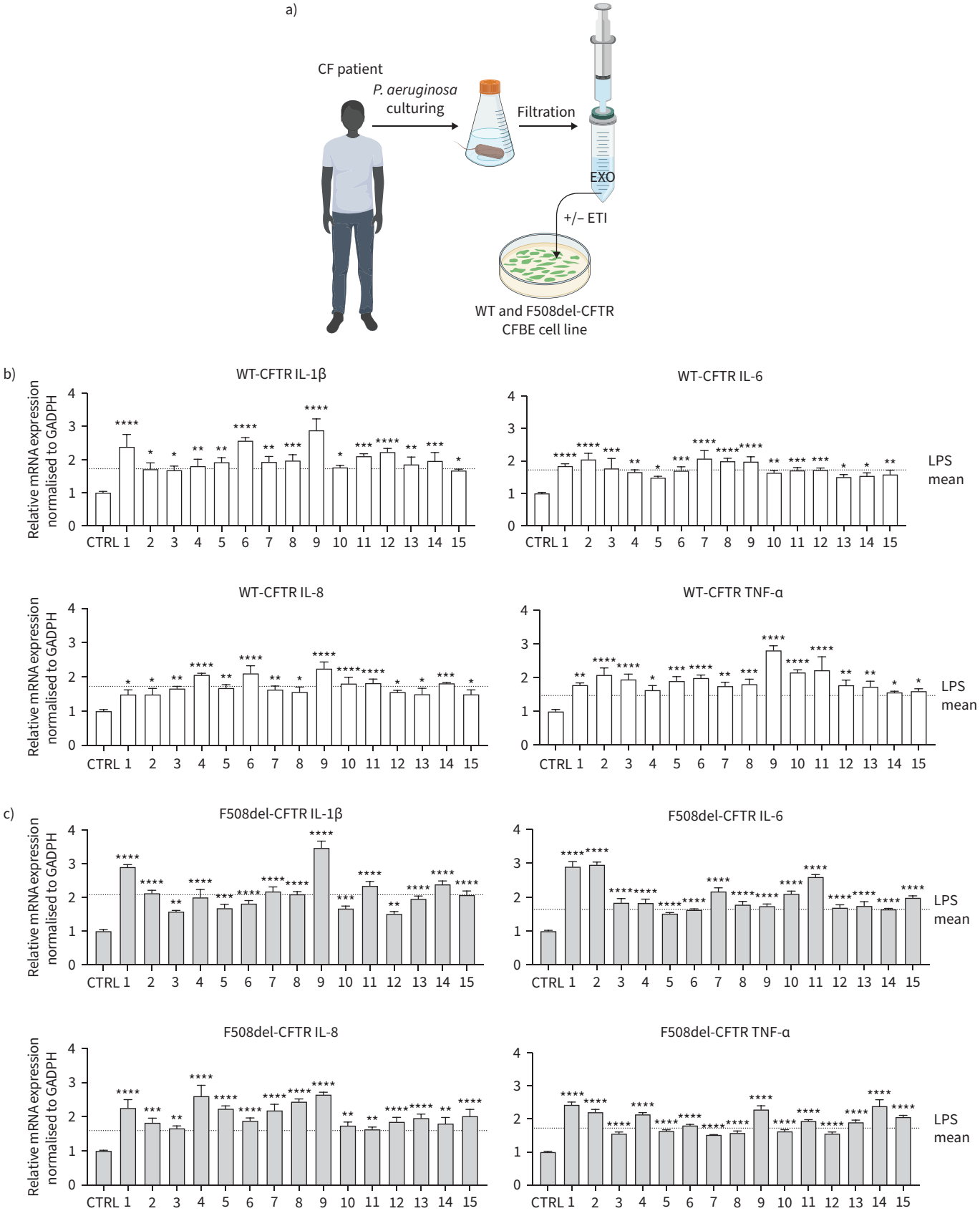
a)
CF patient
P. aeruginosa culturing
Filtration
EXO
+/– ETI
WT and F508del-CFTR CFBE cell line
b)
WT-CFTR IL-1β
WT-CFTR IL-6
WT-CFTR IL-8
WT-CFTR TNF-α
c)
F508del-CFTR IL-1β
F508del-CFTR IL-6
F508del-CFTR IL-8
F508del-CFTR TNF-α
Relative mRNA expression normalised to GADPH
CTRL 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
LPS mean

FIGURE 1 Variable inflammatory response across different clinical exoproducts of *Pseudomonas aeruginosa* obtained from 15 cystic fibrosis (CF) patients in CF bronchial epithelial (CFBE) cell line. a) Cartoon showing the generation of clinical exoproducts (EXO) isolated from CF patients and incubated with CFBE cell line. WT-CFTR b) and c) F508del-CFTR CFBE cells were incubated with clinical exoproducts of *P. aeruginosa* isolated from the sputum of 15 CF patients (1 to 15) for 24 h at 37°C. Total RNA was extracted and quantitative real-time PCR was performed in order to quantify interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α) mRNAs. Bar graphs show the mean±SEM of n=3–4. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. ETI: elexacaftor/tezacaftor/ivacaftor; LPS: lipopolysaccharide, WT: wild-type.

Upon treatment of HNECs with EXO obtained from the *P. aeruginosa* strain from the corresponding pwCF, it was observed that variable expression of IL-1β, IL-6, IL-8 and TNF-α mRNA reduced cytokine levels (figure 5a–d, supplementary figure S3). Interestingly, pretreatment with ETI significantly reduced the EXO-induced cytokines release to control levels but not to the level measured in HNECs from non-CF donors (figure 5a–d). Furthermore, the amount of released IL-8 chemokine was found to inversely correlate with CFTR function measured by Ussing chamber (figure 5e).

Electrophysiological assays are considered the "gold standard" for testing CFTR modulators but are low throughput methods. Therefore, we were prompted to investigate the effect of EXO on nasal epithelial cells differentiated in 96-transwell culture plates by fluorescence-based measurement (fluorescence membrane potential (FMP)) of F508del-CFTR channel activity as described by our previous publications [13, 22]. As shown in figure 6a, nasal epithelial cells from eight pwCF were plated and differentiated in a 96-transwell plate under ALI condition. 12 cultures from a single donor were differentiated simultaneously to investigate EXO effect on CFTR function in the presence or absence of ETI (three technical replicates for each condition). Representative FMP traces of F508del-CFTR function after exposure to vehicle (DMSO) or VX-445+VX-661±EXO for 24 h are shown in figure 6b. A heatmap of F508del-CFTR function in HNECs shows that VX-445+VX-661 treatment rescued F508del-CFTR activity; however, this was decreased by EXO obtained from the *P. aeruginosa* strain from the corresponding pwCF (figure 6c). We observed a strong correlation (r=0.83, p<0.0001) between nasal cultures response to interventions measured by FMP and response measured by Ussing chamber (figure 6d). We then considered four HNEC cultures showing similar ETI response on F508del-CFTR function from figures 4 and 6 (CF3, CF6, CF14 and CF15), and their corresponding EXOs, causing different level of CFTR rescue impairment. We tested the four HNEC cultures after 24 h of treatment with each of the four exoproducts EXO3, EXO6, EXO14 and EXO15 and with ETI. Interestingly, each EXO resulted in a similar reduction of F508del-CFTR function in the four cultures, irrespectively of the different patients' background (figure 6e–f).

P. aeruginosa *exoproducts trigger a slight increase of ROS levels in CF airway epithelial cells reduced by ETI treatment*

By using an ROS-sensitive fluorescence probe, we found that ROS levels were significantly higher in F508del-CFTR compared to WT-CFTR in both CFBE and HNECs (figure 7a,c). Interestingly, ETI treatment significantly decreased the ROS levels, while EXO treatment increased them (figure 7b,d). Then, we analysed the gene expression of Nrf-2 and Nrf-2 regulated proteins involved in detoxification processes (NAD(P)H quinone dehydrogenase 1 (NQO1), glutamate-cysteine ligase modifier (GCLM) and heme oxygenase 1 (HO-1) in HNECs from non-CF, pwCF bearing F508del mutation (ETI responder) and pwCF bearing ETI-resistant mutations): a dramatic decrease of mRNA expression of Nrf2, NQO1, GCLM and HO-1 in pwCF compared to non-CF was observed with no significant difference between ETI responder and nonresponder (supplementary figure S4).

ETI-induced reduction of cytokines and ROS levels is CFTR-dependent in primary nasal cells

To understand whether the reduction of proinflammatory cytokines and ROS levels following ETI treatment was dependent on the recovery of CFTR activity, we treated with ETI for 24 h primary nasal cells from pwCF bearing mutations not or poorly rescued by ETI (E585X/E585X-CF16; R347P/R347P-CF17; E585X/G542X-CF18; R553X/Dele2,3-CF19; G542X/N1303K-CF20). As expected, the CFTR function was not rescued by ETI except for CF17 which showed limited rescue (figure 8a–c). We observed a marked EXO-dependent upregulation of ROS and cytokines (IL-1β, IL-6, IL-8 and TNF-α) production measured as mRNA and protein levels that did not change upon ETI treatment (figure 8d–f).

Discussion

In this study, we characterised the effect of different clinical EXO of *P. aeruginosa* clinical strains obtained from 15 pwCF on the inflammatory response, ROS levels and CFTR function measured in CFBE cells and HNECs. Interestingly, we observed that the functional rescue of F508del-CFTR by the triple combination of CFTR modulators, ETI, was variably impaired by the different EXOs in both CFBE and

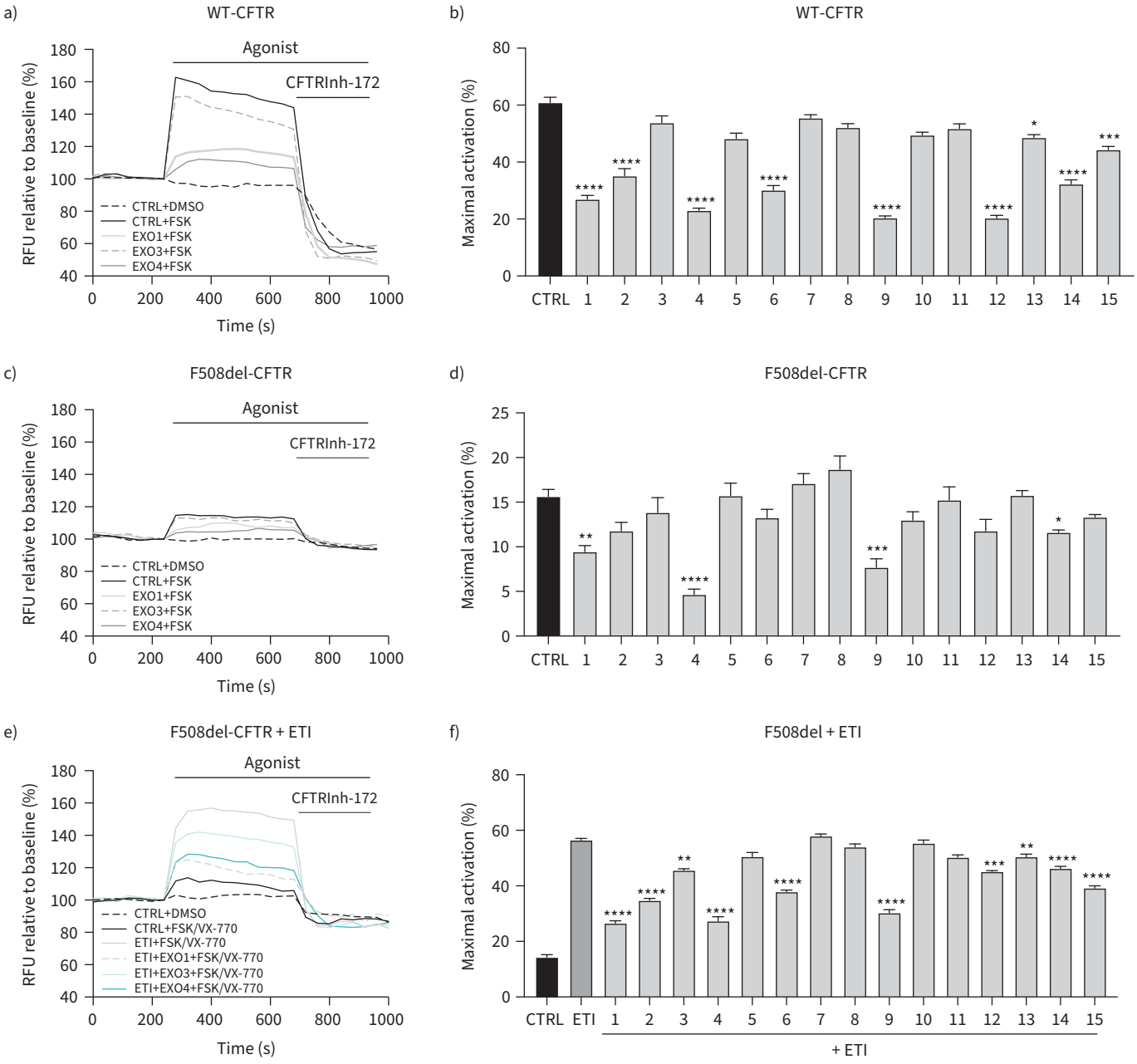

FIGURE 2 Clinical exoproducts of *Pseudomonas aeruginosa* reduced CFTR function dependent on clinical strain in cystic fibrosis (CF) bronchial epithelial (CFBE) cell line. Representative traces of a) WT-CFTR-, c,e) F508del-CFTR-dependent chloride efflux in CFBE cells using fluorescence membrane polarisation (FMP) assay. Cells were treated for 24 h with lysogeny broth (CTRL), clinical exoproducts (EXO) of *P. aeruginosa* from 15 CF patients in presence or absence of 3 µM VX-661+3 µM VX-445 when reported. Bar graphs show the mean±SEM of maximal activation of b) WT-CFTR, d) F508del-CFTR, f) F508del-CFTR treated with 0.1% DMSO, 3 µM VX-661+3 µM VX-445 in CFBE cells after stimulation by 1 µM FSK (for WT-CFTR) and 10 µM FSK+1 µM VX-770 (for F508del-CFTR) (n=3–4 biological replicates with 4 technical replicates each). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. ETI: elexacaftor/tezacaftor/ivacaftor; RFU: relative fluorescence units; WT: wild-type.

HNECs. Additionally, we demonstrated a donor-specific variability in the EXO-induced inflammatory response and ROS levels in the HNECs, partially counteracted by ETI pretreatment.

Previous studies by the Brochiero's group demonstrated that 24 h exposure to exoproducts of *P. aeruginosa* isolated from one pwCF reduced WT-CFTR activity and corrector-mediated F508del-CFTR activity in CFBE cells and primary airway epithelial cells [9]. Moreover, the authors demonstrated that EXO decreased the CFTR protein expression by enhancing protein degradation and reducing protein

a)

Patient	Age years	Genotype	SC mmol·L^{-1}		FEV_1 % pred		LCI		Microbiology
			Before Kaftrio	After Kaftrio	Before Kaftrio	After Kaftrio	Before Kaftrio	After Kaftrio	
1/M	12	F508del/G542X	108	75	102	116	10	6.3	PA
2/F	12	F508del/G542X	95	83	70	96	11.8	8.7	PA
3/F	6	F508del/S549R	95	82	114	142	5.6	6.7	PA
4/M	3	F508del/N1303K	120				12.3		PA MDR
5/M	6	F508del/W1282X	84	62	89	88	5.9	7.6	PA
6/M	9	F508del/G85E	115		93		11.5		PA
7/F	11	F508del/L732X	69	36	98	105	7.5	6.9	PAM, PAW
8/F	27	F508del/L997F	88	25	97	121	7.3	7.4	PA, PAW
9/M	12	F508del/E193K	88	8	69	72	11.7	12.3	PAM
10/F	38	F508del/CFTRdele2,3	93		82		12.6		PAM
11/F	3	F508del/3878delG	106		102		13		PA
12/F	23	F508del/4016insT	88	51	75	79	9.2	9.8	PA
13/F	21	F508del/R785X	109	61	67	68	14.8	15.4	PA MDR, PAM MDR
14/F	34	F508del/L1077P	72	35	42	49	12.1	9.8	PAM
15/F	20	F508del/dele22-23	83	28	54	58	15.1	11.2	PAM MDR
Mean±SEM			89±3.79	49.63±7.56	79.73±6.65	90.36±8.65	10.09±0.99	9.28±0.84	

b)

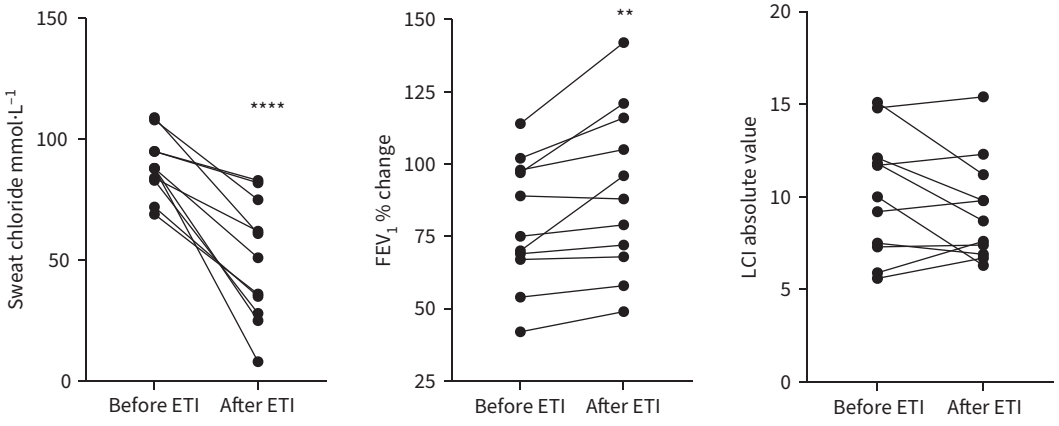

FIGURE 3 Clinical data of brushed nasal cell donors. a) All 15 cystic fibrosis (CF) patients are represented by numbers, which correspond to data points throughout the text and figures. SC: sweat chloride, FEV_1 % pred: % predicted forced expiratory volume in 1 s; LCI: lung clearance index; PA: *Pseudomonas aeruginosa*; PA MDR: *P. aeruginosa* multidrug resistant; PAM: *P. aeruginosa* mucoid isolate; PAM MDR: *P. aeruginosa* mucoid isolate multidrug resistant; PAW: *P. aeruginosa* wrinkled isolate. b) Graphs show the clinical data (sweat chloride, FEV_1 and LCI) before and after 6 months of Kaftrio treatment in 11 CF patients. ETI: elexacaftor/tezacaftor/ivacaftor. **p<0.01; ****p<0.0001.

synthesis. Furthermore, the Stanton's group found that exposure to both laboratory strains and six clinical *P. aeruginosa* strains significantly reduced Lumacaftor+Ivacaftor-mediated F508del-CFTR function in both F508del-CFTR CFBE cells and primary bronchial epithelial cells from F508del/F508del pwCF [10]. All together, these data suggest that CFTR corrector efficacy may be affected by *P. aeruginosa* infection and may explain why lumacaftor/ivacaftor has had modest efficacy in clinical trials [23]. Despite the novel triple combination elexacaftor–tezacaftor–ivacaftor (commercial name: Trikafta/Kaftrio) has shown dramatic improvement in lung function (~+14% FEV_1) in patients carrying at least one *F508del* allele, the overall clinical effect size remains variable [3]. Interestingly, these positive results in preclinical studies of ETI in primary airway epithelial cells from pwCF translate to the clinical outcomes [3], including our data (supplementary figure S3). Therefore, we were prompted to understand if this patient-to-patient variability could be due to the infection conditions in *ex vivo* patient-derived tissues. To mimic the chronic infection/inflammatory CF airway condition, we treated the cells with clinical exoproducts of *P. aeruginosa* [24], rather than live bacteria, isolated from 15 pwCF heterozygous for *F508del*. Interestingly, we found a variable reduction in the WT-CFTR CFBE cells, F508del-CFTR and F508del-CFTR rescued by ETI function in both CFBE cells (figure 1) and HNECs infected with the clinical EXO isolated from the corresponding donor (figure 4). Since airway epithelial cells were chronically treated only with the

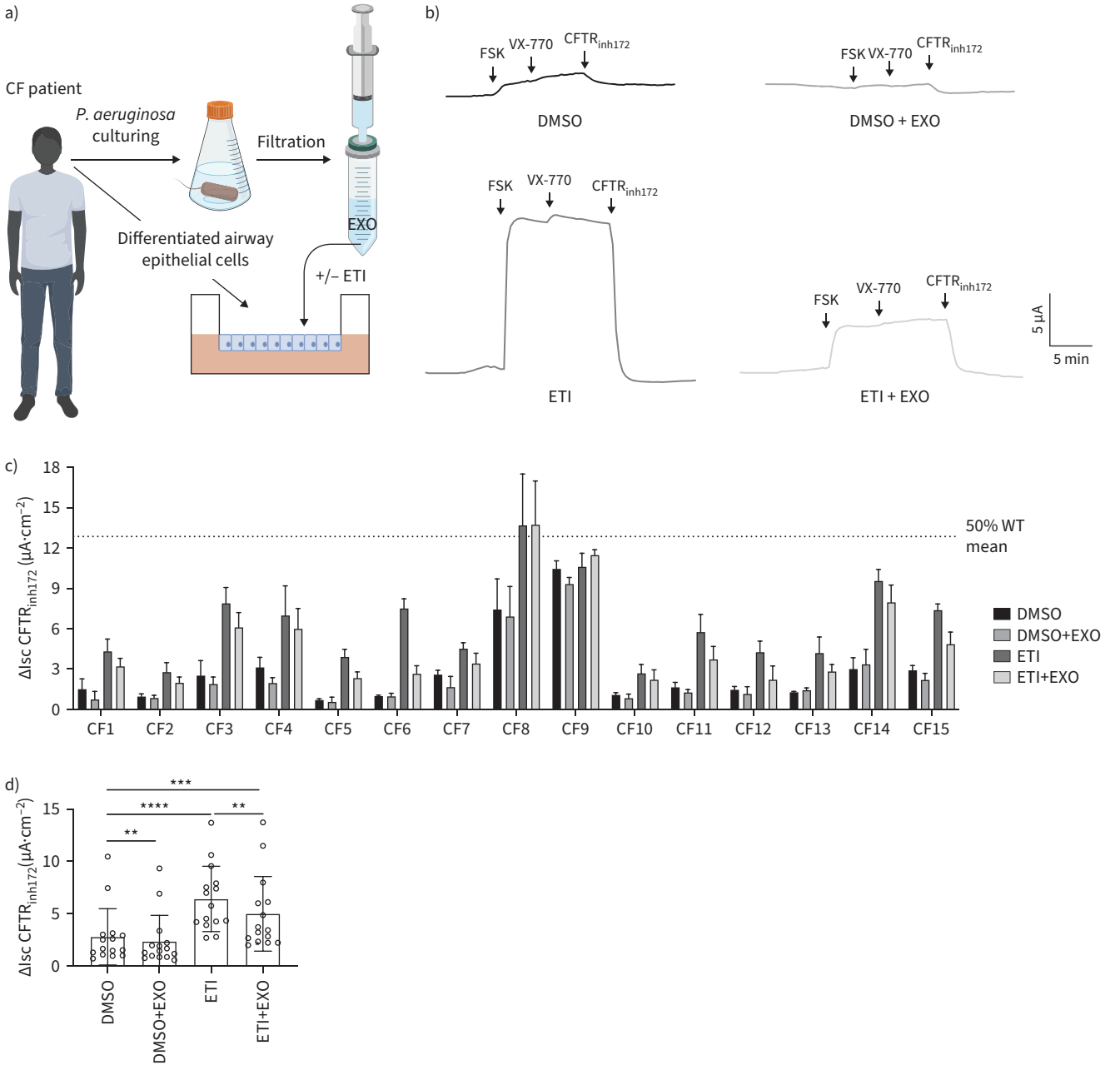

FIGURE 4 Nasal epithelial cultures from 15 cystic fibrosis (CF) patients exhibit differential phenotypic response to VX-445/VX-661/VX-770 in a clinical strain of *Pseudomonas aeruginosa*. a) Cartoon showing the generation of clinical exoproducts (EXO) isolated from CF patients and incubated with primary nasal epithelial cells (HNECs) from the corresponding donor. b) Representative tracings show Ussing chamber measurements of CFTR function in HNEC cultures treated for 24 h with 0.1% DMSO, 3 µM VX-661+3 µM VX-445±clinical exoproducts of *P. aeruginosa* (EXO) isolated from the corresponding patient. c) Bar graph shows the amplitude of the current blocked by 10 µM $CFTR_{inh172}$ ($\Delta Isc_{inh-172}$) after CFTR stimulation with 10 µM FSK+1 µM VX-770 measured in HNEC cultures from each donor. d) Bar graph shows the mean of the amplitude of the current blocked by 10 µM $CFTR_{inh172}$ ($\Delta Isc_{inh-172}$) measured in 15 CF patients. (n=2 technical replicates for each donor). ETI: elexacaftor/tezacaftor/ivacaftor. **p<0.01; ***p<0.001; ****p<0.0001.

two correctors (elexacaftor+tezacaftor), one limitation of the current work is that clinical strains of *P. aeruginosa* may not decrease CFTR activity to the same degree as in the presence of chronic potentiator (ivacaftor). Despite that we specifically isolated *P. aeruginosa*, due to the potential co-infection of *P. aeruginos*a with other pathogens, the clinical strains differences that we observed could be due to the multiple infection. As pwCF are chronically colonised by *P. aeruginosa*, these observations may contribute to explain the variable response effect of ETI on FEV_1% in pwCF. In fact, despite the improvement of

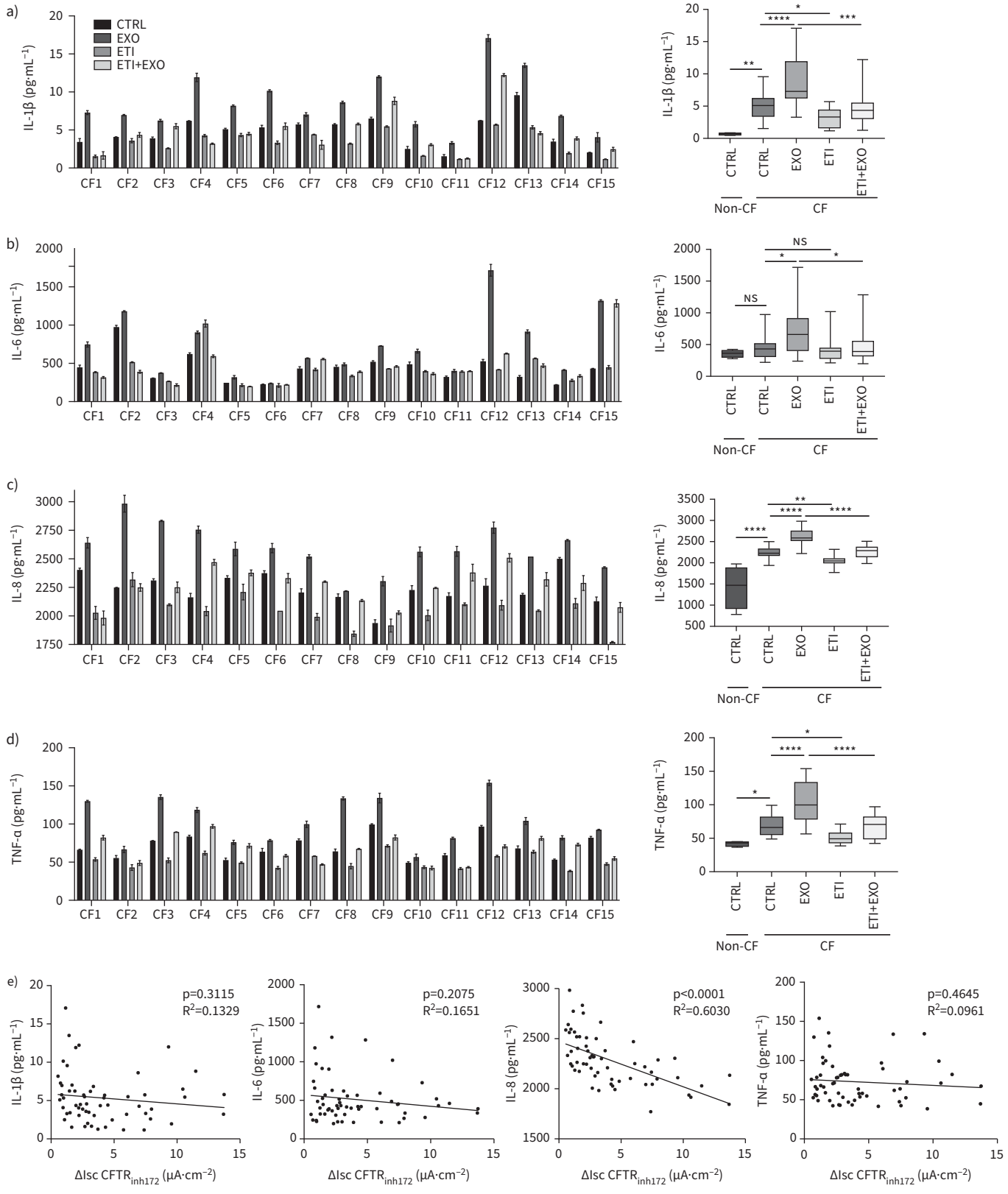

FIGURE 5 Inflammatory response is clinical strain of *Pseudomonas aeruginosa*-dependent and is ameliorated by elexacaftor/tezacaftor/ivacaftor (ETI) treatment. a) Interleukin (IL)-1β, b) IL-6, c) IL-8 and d) tumour necrosis factor-α (TNF-α) secretion after stimulation with lysogeny broth (CTRL), clinical exoproducts of P. *aeruginosa* (EXO) isolated from the corresponding donor, 3 μM VX-661+3 μM VX-445+1 μM VX-770 (ETI)±(EXO) for 24 h in primary nasal epithelial cells from four non-cystic fibrosis (CF) donors and 15 CF patients (n=3). (e) Pearson correlation between released cytokines (pg·mL^{-1}) and CFTR activity ($\Delta Isc_{inh-172}$ μA·cm^{-2}). NS: nonsignificant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

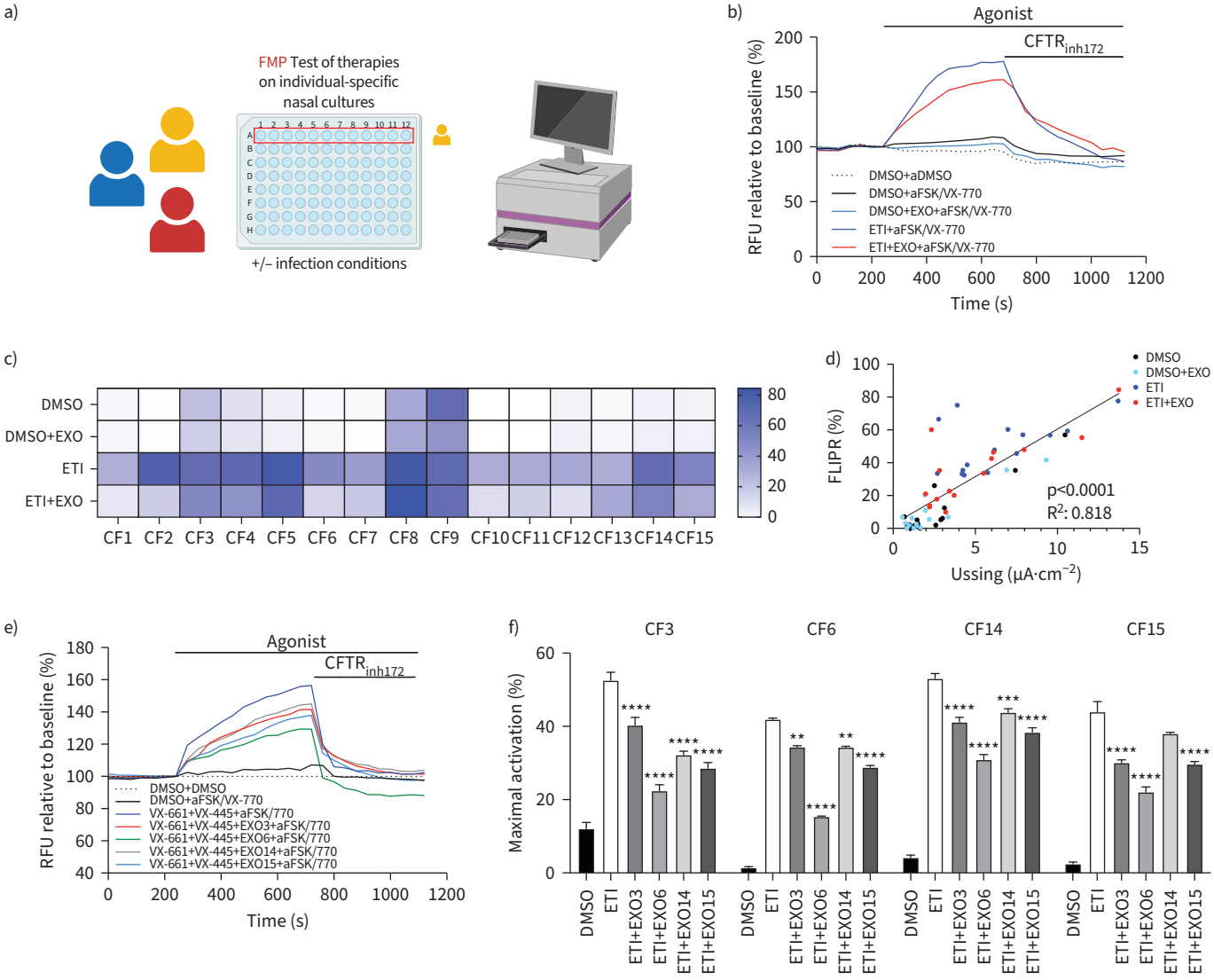

FIGURE 6 Elexacaftor/tezacaftor/ivacaftor (ETI)-rescued F508del-CFTR activity is variably downregulated strain-dependent of *Pseudomonas aeruginosa* in primary nasal epithelial cells by high-throughput fluorescence-based assay. a) Cartoon showing nasal epithelial cells cultured in 96-transwell plate, treated with CFTR modulators in presence or absence of infection stimuli and then tested for apical CFTR activity by fluorescence membrane polarisation assay (FMP) using a plate reader. b) Representative traces of F508del-CFTR-dependent chloride efflux in HNEC cultures by FMP. Cells were treated for 24 h with 0.1% DMSO, 3 µM VX-661+3 µM VX-445±clinical exoproducts of *P. aeruginosa* (EXO) isolated from the corresponding patient. c) Heatmap of peak responses after 10 µM FSK+1 µM VX-770 stimulation generated from HNEC cultures. The response size is colour coded as shown in the side bar, with blue representing the highest response and white the lowest response. (n=3 technical replicates.) d) Correlation between mean donor-specific activation measured using FMP (expressed as % of maximal activation) and mean donor-specific activation measured in the Ussing chamber ($\Delta Isc_{inh\text{-}172}$ µA·cm^{-2}). e) Representative traces of F508del-CFTR activity in HNECs treated for 24 h with 0.1% DMSO, 3 µM VX-661+3 µM VX-445±clinical exoproducts of *P. aeruginosa* (EXO) isolated from patient CF3 (EXO3), CF6 (EXO6), CF14 (EXO14) and CF15 (EXO15). f) Bar graphs show the mean±SEM of maximal activation after 10 µM FSK+1 µM VX-770. (n=3 technical replicates.) Statistical analysis compared to ETI control. **p<0.01; ***p<0.001; ****p<0.0001. RFU: relative fluorescence units.

respiratory outcomes observed in CF patients treated with highly effective modulators, the airway inflammation and bacterial colonisation still persist [25–28].

Recently, we developed a high-throughput FMP-based assay to measure CFTR activity in patient-derived tissues [20, 22, 29, 30]. Therefore, we performed FMP to measure F508del-CFTR activity in HNECs cultured in 96-transwell plates infected with clinical EXO isolated from the corresponding donor (figure 6). The results obtained by FMP assay recapitulated the patient-specific responses to CFTR modulators measured by the Ussing chamber assay, which is considered the gold standard for CFTR correctors

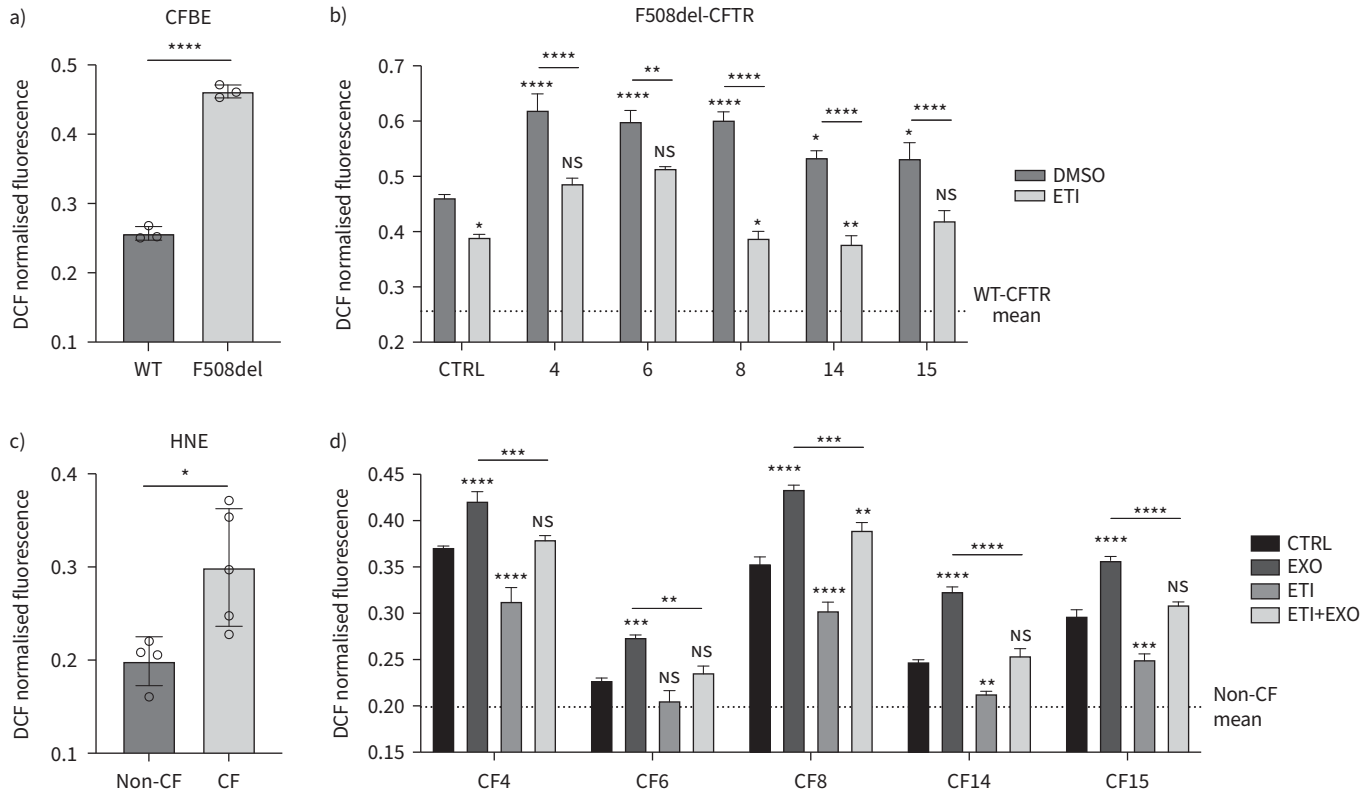

FIGURE 7 Clinical exoproducts of *Pseudomonas aeruginosa* increased the intracellular levels of the reactive oxygen species (ROS) and were reduced by elexacaftor/tezacaftor/ivacaftor (ETI) treatment in both cystic fibrosis (CF) bronchial epithelial (CFBE) cells and primary nasal epithelial cells. Cells were treated with clinical exoproducts of *P. aeruginosa* isolated from the sputum of five CF patients (1 to 15) ±3 µM VX-661+3 µM VX-445+1 µM VX-770 (ETI) for 24 h at 37°C. ROS levels were measured by 10µM o5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (H2DCFDA)-based assay. a,b) Bar graphs show the mean±SEM of ROS levels in wild-type (WT) and F508del-CFTR CFBE cells (n=3). c) Bar graph shows the mean±SEM of ROS levels in primary nasal cells from four non-CF donors and five CF patients. d) Bar graph shows the mean±SEM of ROS levels in nasal epithelial cells from five CF patients. Cells were treated for 24 h with 0.1% DMSO, 3 µM VX-661+3 µM VX-445±clinical exoproducts of *P. aeruginosa* (EXO) isolated from the corresponding patient. (n=3 technical replicates for each patient.) *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. DCF: 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA); WT: wild-type.

screening. These findings further support the use of this method to high-throughput screening CFTR modulators in the presence and absence of infection conditions as a complementary approach for personalised therapeutical strategy in primary nasal cells. Moreover, using FMP assay in nasal cells from four pwCF, all of them treated with four EXOs, we observed that the variable reduction of ETI-mediated F508del-CFTR function is EXO-dependent and not patient's background (figure 6e–f). Further studies, performed on an increased number of pwCF, are needed to confirm that a better prediction of the clinical response to CFTR modulators could be achieved if the functional studies are performed in patient-derived cells infected with *P. aeruginosa* EXOs isolated from the same donor.

P. aeruginosa infections in the lung of pwCF have long been associated with an increased inflammatory response. To model the inflammation, it is common to use LPS or laboratory strain of *P. aeruginosa* (*i.e.* PAO1). However, in the current study we demonstrated high variability in the inflammatory cytokine levels stimulated by different clinical EXOs of *P. aeruginosa* isolated from 15 pwCF in both CFBE and HNECs (figures 2 and 6, supplementary figure S4). Our data suggest that *in vitro* inflammation studies should also be performed using patient-derived tissues infected with clinical *P. aeruginosa* isolated from the corresponding donor.

It has been previously shown that tezacaftor/ivacaftor and lumacaftor/ivacaftor downregulates the inflammation [24, 31]. On the other hand, the Stanton's group demonstrated that lumacaftor or lumacaftor/ivacaftor had no effect on IL-6 and IL-8 secretion in CFBE cells treated with vehicle or *P. aeruginosa* [10]. Therefore, we investigated the effect of ETI on inflammatory response in HNECs infected with

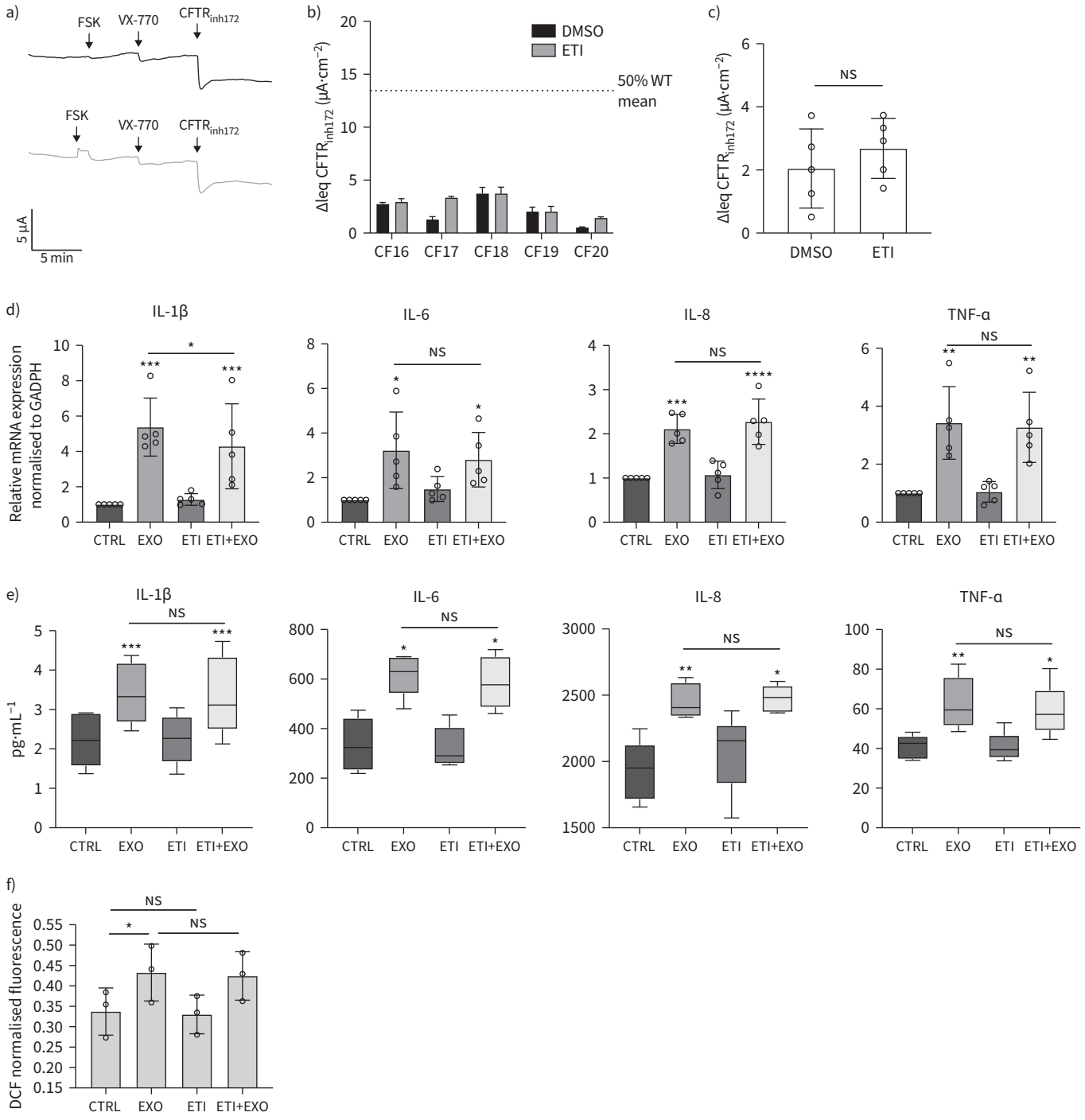

FIGURE 8 ETI treatment did not decrease proinflammatory released cytokines and reactive oxygen species (ROS) levels in primary nasal epithelial cells from cystic fibrosis (CF) patients bearing elexacaftor/tezacaftor/ivacaftor (ETI)-resistant mutations. a) Representative tracings show Ussing chamber measurements of CFTR function in primary human nasal epithelial cell (HNEC) cultures from five CF patients bearing E585X/E585X (CF16), R347P/R347P (CF17), E585X/G542X (CF18), R553X/Dele2,3 (CF19), G542X/N1303 K (CF20). Cells were treated for 24 h with 0.1% DMSO, 3 µM VX-661+ 3 µM VX-445. b) Bar graph shows the amplitude of the current blocked by 10 µM $CFTR_{inh172}$ ($\Delta Isc_{inh-172}$) after CFTR stimulation with 10 µM FSK+1 µM VX-770 measured in HNEC cultures from each donor. c) Bar graphs show the mean of the amplitude of the current blocked by 10 µM $CFTR_{inh172}$ ($\Delta Isc_{inh-172}$) measured in five CF patients. d) Interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α) mRNA levels and e) secretion after stimulation with lysogeny broth (CTRL), clinical exoproducts of *Pseudomonas aeruginosa* (EXO), 3 µM VX-661+3 µM VX-445+1 µM VX-770±(EXO) for 24 h in HNEC cultures from five CF patients. f) Bar graph shows the mean±SEM of ROS levels in nasal epithelial cells from three CF donors. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. DCF: 2′,7′-dichlorodihydrofluorescein diacetate.

clinical EXOs and found a significant decrease of basal and EXO-induced airway inflammatory markers (*i.e.* IL-8) even though not to non-CF levels (figure 6). Moreover, our study is also in agreement with clinical studies showing anti-inflammatory effects of ETI by reducing the levels of neutrophil elastase, cathepsin G, IL-1β and IL-8 [32–34]. On the other hand, other groups demonstrated that inflammatory mediators that are elevated in CF airways enhance the restoration of CFTR channel function in response to CFTR modulators [35–37]. This discrepancy may be due to the different methods employed: the Ribeiro's group stimulated the primary bronchial cells with the supernatant from mucopurulent material obtained from the airway of excised human CF lungs from pwCF while we stimulated primary nasal cells with clinical exoproducts of *P. aeruginosa* from the corresponding CF donor. Considering these complex, inconsistent and potentially harmful effects, the optimal strategy to target residual airway inflammation in people taking Trikafta/Kaftrio remains unclear.

It has been reported that a redox imbalance could be associated to alterations of CFTR function in epithelial cells [38]. In this study, we demonstrated, for the first time, that the basal ROS levels measured in CF primary nasal cells are higher than in non-CF cells (figure 7a). *P. aeruginosa* infection may cause increased ROS levels either endogenously *via* respiration or exogenously by the host immune system or disinfectants [39]. Therefore, we measured ROS levels in HNEC cultures exposed to EXO isolated from the corresponding patient and found variable strain-specific manner ROS levels that were decreased after ETI pretreatment (figure 7c–d). In order to clarify whether the "anti-inflammatory" and "antioxidant" activity shown by ETI is dependent on CFTR rescue (and, thus, CFTR activity), we employed primary nasal cells from pwCF not responsive to ETI (bearing nonsense or splicing mutations). Interestingly, we found that ETI, as expected, did not rescue mutated CFTR and neither decreased cytokines nor ROS levels (figure 8). Therefore, our data suggest that the partial rescue of CFTR function with CFTR modulators is sufficient to reduce the inflammation and redox imbalance thus confirming that the anti-inflammatory and antioxidant activity of ETI is mediated by the rescue of CFTR activity.

It has been recently demonstrated that Nrf2, a transcription factor involved in redox homeostasis and inflammatory signalling, is downregulated in CF human bronchial epithelial (HBE) cells [40]. Moreover, VX-809 and VX-661 rescued Nrf2 mRNA and function in CF HBE cells but not in CF HBE cells after CFTR knockdown by shRNA [41]. In the current study, we confirmed the lower expression of Nrf2 in CF nasal cells compared to non-CF cells, suggesting that dysfunction of Nrf-2 in CF HNECs may play a role in increasing inflammatory cytokines and intracellular ROS. Moreover, the ETI-mediated enhanced gene expression of Nrf2- and Nrf2-regulated genes (*i.e.* NQO1, GCLM, HO-1) in CF-HNEC cultures responding to ETI (and not in those with mutations not responsive to ETI) suggests that the decrease of inflammatory/oxidative status due to ETI treatment is mediated by CFTR rescue. However, ETI did not fully restore Nrf2 to non-CF levels and, consistently, cytokines and ROS levels were still higher than those of a healthy control (figures 6 and 7).

In summary, we observed variable ETI-rescued CFTR function, inflammatory response and oxidative stress in HNECs infected with clinical exoproducts of *P. aeruginosa* strains isolated from the corresponding pwCF. Our *in vitro* study also highlighted for the first time that, by rescuing CFTR function, ETI exerts an anti-inflammatory and antioxidant activity. Collectively, our data strongly suggest that *in vitro* screening of patient-specific response to CFTR modulators, under conditions mimicking infection, could prove to be a valuable tool to better predict the clinical response, aimed to develop more effective personalised therapeutic strategies with the appropriate antibiotics or anti-inflammatory therapies.

Provenance: Submitted article, peer reviewed.

Ethics statement: This study was approved by the Research Ethics Board of Bambino Gesù Hospital (2961/2022) and Giannina Gaslini Hospital (CER 28/2020). All study participants or their guardians signed an informed consent.

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.

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