Effect of Elexacaftor/Tezacaftor/Ivacaftor on airway and systemic inflammation in cystic fibrosis.

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SUPPLEMENTARY METHODOLOGY

Patient Recruitment

A total of 45 patients presenting to Beaumont Hospital Adult CF unit were deemed eligible for the study based on genotype and above limitations. From the initial recruitment visit, patients were excluded if they had known allergy to any of the drug components, exacerbation at time of recruitment or study visits and if unable to produce spontaneously expectorated sputum sample (SS) (Figure S1). Limitations in in-person clinical review and sputum induction due to the COVID pandemic resulted in a reduced number of eligible participants, meaning only patients who could attend in person and produce spontaneously expectorated sputum samples (SS) could be included.

Plasma collection and processing

Blood was collected in lithium heparin tubes and placed immediately on ice. Blood tubes were centrifuged at 875g for 10min at 4°C and the upper layer (plasma) was collected. Plasma aliquots were stored at -80°C for subsequent cytokine analysis and AAT level measurement.

Protease activity assays

Protease activity was assayed using specific peptide substrates as described previously[1]. Briefly, sputum (25 µl) was incubated in 100 mM tris, 125 mM NaCl, 0.05% Igepal CA-630, pH 7.4, at 37 °C for 10 min in the presence of neutrophil elastase substrate, Abz-APEEIMRRQ-EDDnp (20 µM, Peptides Int'l), cathepsin G substrate Abz-EPFWEDQ-EDDnp or proteinase 3 substrate, Abz-VADnVRDRQ-EDDnp. Fluorescent cleavage product was measured by excitation at 320 nm and emission detected at 420 nm using a SpectraMax M3 plate reader (Molecular Devices). Protease concentrations were derived from reaction rates by use of standard curves (0.01 – 10 nM, Elastin Products Co.; Eurogentec).

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Enzyme-linked immunosorbent assay (ELISA)

Protein levels in sputum or plasma were quantified in 100 µl duplicate volumes using Quantikine ELISA kits (Bio-Techne) per manufacturers' recommendations. Levels were quantified following 1:10 – 1:1000 dilutions in phosphate-buffered saline as required.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western Blotting

Western blot analysis was performed on sputum supernatant samples of people with cystic fibrosis (PWCF) for Secretory Leukocyte Protease Inhibitor (SLPI). Protein quantity in sputum samples was measured using a BCA Protein Assay Kit (Pierce®), and absorbance was recorded at 540 nm using a SpectraMax M3 microplate reader (Molecular Devices). Protein samples (30 µg) were loaded onto 15% polyacrylamide gels and transferred to PVDF membrane (Roche). Primary antibody (R&D Systems, AF1274) was incubated overnight at 4°C, then probed with corresponding secondary antibody (Santa Cruz Biotechnology, sc-2020) for 1h at ambient temperature. Purified SLPI (100 ng; 1274-PI, Bio-Techne) was included in a discrete lane as protein standard. Proteins were detected with Immobilon Western Chemiluminescent Substrate (Merck Millipore) using a ChemiDoc™ Imaging System (Bio-Rad).

Statistical Analysis

Significance was tested for using a within-subjects repeated-measures mixed effects model, with Tukeys post-hoc multiple comparison test (Graphpad Prism, version 9.5) for sputum or plasma markers or by two-tailed, unpaired Students t test when comparing between a given timepoint for CF and NCFB cohorts. Normality of data was tested for by Kolgomorov-Smirnov test. Values of $P < 0.05$ were considered significant and multiplicity adjusted P values are reported. Data were log transformed where substantial range affected readability and were analysed by Wilcoxon matched-pairs signed rank test. Significant groupwise differences between categorical variables were estimated using Fishers exact test.

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SUPPLEMENTARY DATA

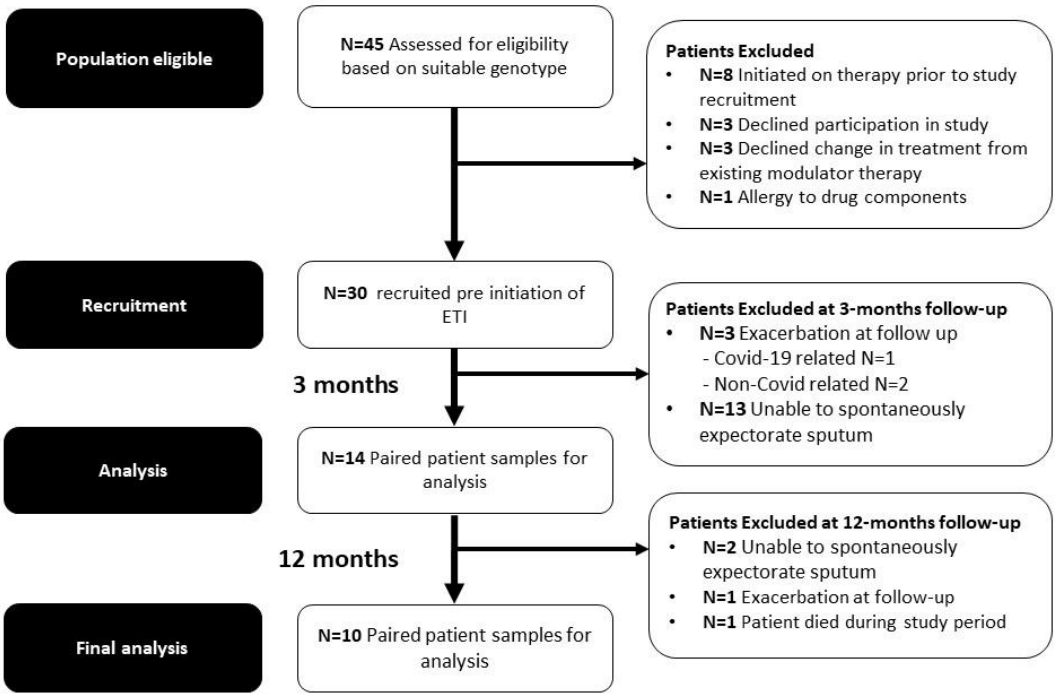

Figure S1: Study flowchart of patients included in this study.

Initial N=45 eligible for study inclusion based on genotype, only N=30 PWCF recruited due to the exclusion criteria highlighted above. At 3 months, only N=14 eligible due to; N=13 unable to produce SS, N=1 Covid-19 related pulmonary exacerbation and N=2 non-Covid-19 related pulmonary exacerbation. At 12-month follow up, N=10 PWCF were included as N=2 unable to produce SS, N=1 pulmonary exacerbation, N=1 died.

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Clinical characteristics of inflammatory lung disease control cohort.

People with non-cystic fibrosis bronchiectasis (NCFB) experience chronic neutrophilic airway inflammation with similar, albeit less severe, presentation to that of cystic fibrosis. Hence, they are an important comparator for studies of putative treatments for airway inflammation in CF. For this study, ten individuals with NCFB who could spontaneously expectorate sputum, and who were matched for lung function (FEV_1 % predicted: 57.87 ± 24.4 versus 61.3 ± 16; p = 0.68), were recruited at study outset (Table S1). Subjects with idiopathic bronchiectasis or with primary ciliary dyskinesia were of normal genotype apart from one individual with PCD who was heterozygous with a V520F variant in *CFTR* that, in the absence of a second *CFTR* mutation, is not disease-causing. Subjects with post-infection bronchiectasis were not routinely genotyped but did not have a clinical profile consistent with CF.

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Table S1. Patient demographics for NCFB cohort	
Cohort	Sputum
Total number	10
Age (years)	46.0 ± 20.8
Sex	
Male	2 (20)
Female	8 (80)
FEV_1	61.30 ± 16.07
BMI (kg/m^2)	25.9 ± 3.8
Aetiology of Bronchiectasis	
Primary Ciliary Dyskinesia	5 (50)
Idiopathic	2 (20)
Post-infection	3 (30)
Pseudomonas colonisation	6 (60)
Hypertonic Saline	6 (60)
Inhaled Corticosteroids	8 (80)
Chronic Inhaled antibiotics	3 (30)
Azithromycin	5 (50)

Data are expressed as number or mean ± standard deviation (%). FEV_1, forced expiratory volume in 1s (% predicted); BMI, body mass index.

Clinical characteristics of PWCF able to produce spontaneously expectorated sputum (SS) at 3-months post-ETI therapy versus PWCF unable to produce SS at 3-months follow-up.

At 3-months post-ETI, 13 patients were excluded from the study as they were unable to produce an SS sample. Table S2 shows the clinical characteristics of the 14 patients able to produce an SS sample versus the 13 unable to produce an SS sample at 3-months post-ETI. Patients unable to produce sputum at 3-months post-ETI had a higher baseline FEV_1 % predicted pre-ETI 71.5 ± 25 versus 44.07 ± 16.93 for those able to provide an SS sample at 3-months and only 38% were chronically colonised with *Pseudomonas aeruginosa* versus 86% of patients able to provide an SS sample at 3-months.

Treatment with azithromycin (AZM) can affect inflammation in certain contexts but did not influence our cohort at baseline; mean circulating IL-6 levels in those who were in receipt of AZM were 230±53 pg/ml and 224±22 pg/ml in those not in receipt of AZM (p=0.79). Similarly, mean IL-1β levels were 441±159 pg/ml and 374±119 pg/ml, respectively (p=0.41).

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Table S2. Baseline demographics of PWCF able to produce spontaneously expectorated sputum at 3-months post-ETI therapy versus PWCF unable to produce SS at 3-months.

	Sputum Producers	Sputum Non-producers	*P* value†
Total number	14	13	
Age (years)	30 ± 5	26.2 ± 7	0.0863
Sex			0.6676
Male	9 (64)	10 (77)	
Female	5 (36)	3 (23)	
Genotype			0.6483
F508del/*F508del*	10 (71)	11 (85)	
F508del/Minimal Function	4 (29)	2 (15)	
FEV_1	44.07 ± 16.9	70.13 ± 24	0.0018
BMI (kg/m^2)	22 ± 2	23.45 ± 2.2	0.1118
Pseudomonas colonization	12 (86)	5 (38)	0.0183
Previous CFTR modulator use			0.9006
Tezacaftor/Ivacaftor	6 (43)	6 (46)	
Lumacaftor/Ivacaftor	4 (29)	3 (23)	
None	4 (29)	4 (31)	
Azithromycin	8	6	0.7064
Dornase alfa	14	13	0.9999
Hypertonic saline	10	8	0.6946
Inhaled corticosteroids	8	7	0.9999
Chronic inhaled antibiotics	11	5	0.0542

Data are expressed as number or mean ± standard deviation.
FEV_1, forced expiratory volume in 1s (% predicted); BMI, body mass index.
† Fishers exact test

Clinical characteristics of circulating inflammatory marker control cohort.

We further examined systemic inflammation in the sub-group of PWCF who had advanced lung disease or features associated with worse prognosis at the time of recruitment (Figure 2 in Main Text). Patients were deemed as having advanced lung disease based on $FEV_{1pp} \leq 40\%$ or those with a CF-ABLE score ≥ 5, long term oxygen therapy (LTOT) and/or non-invasive ventilation (NIV) requirement. Features associated with worse prognosis included frequent pulmonary exacerbations or infection with difficult to manage organisms[2,3]. To evaluate systemic inflammation in an inflammatory lung disease control we evaluated inflammatory markers in a separate NCFB cohort (n=18, Figure 2, Table S3). This cohort was of older age, but comparable in BMI, carriage of microorganisms including *P. aeruginosa* and with similar medication history. Circulating markers in PWCF with advanced disease were compared with people with NCFB once begun on ETI therapy (main text, figure 2).

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Table S3. Clinical characteristics of NCFB cohort for circulating inflammatory marker study.	
Number	18
Age (years)	53.94 ± 11.42
Sex	
male	9
female	9
FEV_1	56.51 ± 15.49
BMI (kg/m^2)	24.3 ± 4.5
Aetiology of bronchiectasis	
Primary ciliary dyskinesia	5
Childhood infection	4
Mycobacterial infection	1
COPD	3
Connective tissue disorder	1
Idiopathic	1
*Pseudomonas aeruginosa**	10
Prior NTM[†]	1
Prior ABPA[†]	0
Current smokers	1
Former smokers	9
Concomitant medications	
Hypertonic saline	3
Recombinant DNAse	1
Chronic inhaled corticosteroids[‡]	3
Chronic inhaled antibiotics[‡]	10
Maintenance azithromycin[‡]	9

Data expressed as number (%) or mean ± Standard Deviation
FEV_1 – forced expiratory volume in 1s (% predicted); BMI – body mass index; NTM – non-tuberculous *mycobacteria;* COPD – chronic obstructive pulmonary disease
* positive on most recent culture of sputum or bronchoalveolar lavage fluid
[†]treated within previous two years
[‡] Defined as receiving ongoing treatment for ≥2 weeks with oral azithromycin while at clinical baseline (i.e. not for acute infective exacerbation)

References

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