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Alexandre Cammarata-Mouchtouris October 29, 2024

People with cystic fibrosis on elexacaftor-tezacaftor-ivacaftor with high sputum neutrophil elastase exhibit worse pulmonary function and pro-inflammatory airway milieu

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People with cystic fibrosis on elexacaftor-tezacaftor-ivacaftor with high sputum neutrophil elastase exhibit worse pulmonary function and pro-inflammatory airway milieu

By Alexandre Cammarata-Mouchtouris

Abstract

Background: Cystic fibrosis (CF) is a genetic disorder characterized by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), leading to multi-organ pathology and progressive respiratory failure. Neutrophil elastase (NE) activity, a critical component of the inflammatory response in CF, has been implicated in disease severity. The highly effective modulator therapy (HEMT) elexacaftor-tezacaftor-ivacaftor (ETI) has shown significant improvements in pulmonary function, but its impact on inflammation remains variable among patients.

Methods: We conducted a cross-sectional, single-center study involving 41 clinically stable individuals with CF, including 32 on ETI therapy and 9 not receiving any modulator treatment (NoHEMT). Sputum samples were analyzed for NE and myeloperoxidase (MPO) activities, pro-inflammatory cytokines, chemokines, and metabolites. Lung function was assessed using %FEV1_pred at collection date (GLI). Multivariate multiple regression models were employed to explore the relationships between NE activity and primary outcomes, with adjustments for multiple comparisons using the Bonferroni correction.

Results: NE activity was significantly higher in the NoHEMT group compared to the ETI group ($p=0.0090$). Within the ETI group, a bimodal distribution of NE activity was observed, distinguishing two sub-populations: high NE activity (NE^{Hi}) and low NE activity (NE^{Lo}). NE^{Hi} subjects exhibited significantly increased MPO activity ($p=0.0002$) and elevated levels of several cytokines, including IL-1β, IL-6, IL-10, TNF-α, and VEGF-A. Metabolomic analysis revealed 107 metabolites significantly associated with high NE activity, indicating broad neutrophil-driven pro-inflammatory metabolome disruption. Lung function was significantly lower in NE^{Hi} subjects both before and after ETI initiation ($p=0.0117$), with NE^{Hi} subjects showing worse lung function that was not fully rescued by ETI therapy.

Conclusions: Our findings highlight the heterogeneity in inflammatory responses among PwCF on ETI therapy. High NE activity is associated with increased inflammation and worse lung function, suggesting that additional inflammation-targeted therapies may be necessary to maximize the benefits of ETI in CF patients with advanced pro-inflammatory manifestations of lung disease. Further research is needed to understand the mechanisms underlying differential responses to ETI and to develop tailored therapeutic strategies.

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Introduction

Cystic fibrosis (CF) is a heritable condition caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), resulting in multi-organ pathology including the gastrointestinal tract, pancreas, lungs, reproductive organs, and others.[1,2] Progressive respiratory failure remains the leading cause of death among people with CF (PwCF), characterized by airways obstruction, polymicrobial infections, and chronic neutrophilic inflammation.[2]

A critical component of the inflammatory response in CF is the activity of neutrophil elastase (NE), a protease released by activated neutrophils. Elevated levels of NE activity have been implicated in the pathogenesis of CF lung disease, contributing to tissue destruction, mucus hypersecretion, and impaired bacterial clearance. Recent studies have highlighted the significance of NE activity as a biomarker for disease severity in CF. High levels of NE activity are associated with worse clinical outcomes, including accelerated decline in lung function and increased frequency of pulmonary exacerbations. Conversely, low levels of NE activity are indicative of a less aggressive inflammatory response and better overall prognosis. For instance, a study demonstrated that surface-bound NE activity on sputum neutrophils is significantly elevated in CF patients compared to healthy controls, correlating with reduced lung function and increased inflammation.[3] Similarly, a review discussed the role of NE in driving the inflammatory cascade and its impact on lung tissue integrity in CF.[4]

The highly effective modulator therapy (HEMT) cocktail elexacaftor-tezacaftor-ivacaftor (ETI, marketed by Vertex Pharmaceuticals as Trikafta™ in the United States and Kaftrio™ in the

European Union) is indicated for the treatment of PwCF who have at least one F508del mutation in the *CFTR* gene, accounting for approximately 90% of ≥2-year-old PwCF, though access may be limited by drug cost and potential toxicity.[1] Use of ETI is associated with rapid increases in pulmonary function and decreased airways obstruction.[5-7] By enhancing CFTR chloride channel activity, ETI is anticipated to increase airway hydration, consistent with observations of decreased mucus viscosity and increased airway clearance.[8] Notably, previously routine sputum expectoration ceases for many individuals after ETI initiation,[9] and emerging evidence suggests ETI has meaningful anti-inflammatory benefits. In a longitudinal study, ETI decreased sputum NE and IL-1β, while having less impact on other inflammatory markers such as IL-8, IL-6, TNF-α, cathepsin G, and proteinase-3.[10] Another longitudinal study showed anti-inflammatory effects in sputum across the aforementioned range of targets, except IL-6 and TNF-α (not analyzed), with resolution to levels comparable to non-CF bronchiectasis controls.[9] A third longitudinal study found sputum NE, IL-1β, and IL-8 all decreased a month after initiation.[11] ETI has also been observed to decrease sputum and bronchoalveolar lavage neutrophils,[9,12] and systemic inflammatory mediators such as IL-6, IL-8, IL-17a, and C-reactive protein, as well as diminishing the predominance of neutrophils.[9,13] Overall, despite the significant advancements brought by HEMT such as ETI, which has shown remarkable improvements in pulmonary function and reduction in airway obstruction, there remains a critical need to address the underlying inflammatory processes in CF.

While ETI has demonstrated anti-inflammatory benefits, including reductions in sputum NE and other inflammatory markers, the heterogeneity in patient responses suggests that not all individuals experience the same degree of benefit. This variability underscores the importance of further

investigating the factors that contribute to differential responses to ETI. To better understand this heterogeneity, we conducted a cross-sectional, single-center study of sputum from adults with CF taking ETI, compared to controls receiving no HEMT. Our analysis focused on the activities of NE and myeloperoxidase (MPO), pro-inflammatory cytokines and chemokines, and metabolites in airway sputum supernatant, alongside pulmonary function testing and other clinical data. The findings revealed a bimodal distribution of NE activity in the sputum of individuals using ETI, highlighting a subset of patients with persistently high NE activity. This subgroup exhibited broad evidence of increased neutrophilic inflammation and metabolic disruption, as well as worse lung function both prior to and following ETI initiation. These results suggest that inflammation-targeted therapies are needed to maximize the benefits of ETI in CF patients with advanced pro-inflammatory manifestations of lung disease.

Chapter 1: Differences in sputum inflammatory profiles of PwCF taking ETI or no HEMT

Study demographics

We analyzed 41 clinically stable individuals, consisting of 32 PwCF undergoing ETI therapy ("ETI") and 9 not receiving any modulator treatment ("NoHEMT"). Summary demographics and clinical characteristics are provided in **Table 1**. Subjects are divided according to whether they were receiving ETI or no modulator therapy at the time of sputum donation. Sputum donors must have been taking ETI for at least 3 months to be considered part of the ETI group. Because of limited sputum volumes, not all assays were conducted on every sample; deviations in sample size for specific assays are noted accordingly. The groups did not significantly differ in age, sex, race, lung function, BMI, pancreatic sufficiency, or use of insulin therapy. However, the distribution of CFTR genotypes in PwCF taking ETI trended toward more F508del homozygosity and less non-F508del homozygosity (p=0.06, Fisher's exact test). This result could be anticipated based on requirements of ETI eligibility.[14,15] In summary, a trend toward different CFTR genotype distributions distinguished these groups, but they were otherwise highly comparable.

We did a correlation analysis between NE activity and lung function for the two groups of PwCF (**Table 2**). In the NoHEMT group, the unadjusted correlation analysis revealed a moderate negative correlation between NE activity and lung function (%FEV1_pred), with a correlation coefficient of -0.471 (p=0.201). However, this relationship was not statistically significant. After adjusting for confounders, including age, gender, level of MPO activity, and CFTR genotype, the correlation coefficient changed to 0.699 (p=0.189), indicating a strong positive correlation, though still not statistically significant. In contrast, the group on ETI therapy showed a significant moderate negative correlation in the unadjusted model, with a correlation coefficient of -0.436 (p=0.018).

This negative correlation persisted and slightly strengthened in the adjusted model, with a correlation coefficient of -0.477 (p=0.029), remaining statistically significant.

Differences in sputum inflammatory profiles of PwCF taking ETI or no HEMT

We sought to compare PwCF taking ETI to individuals who were not taking HEMT. First, we analyzed cytokines as important mediators of inflammation. Across 18 cytokines analyzed in total, none were significantly different between the two groups after adjusting for multiple comparisons (**Figure 1**). We also considered NE and MPO activities, as these are critical effector molecules of neutrophils that modify the airway environs in CF. NE activity was significantly increased in PwCF not taking HEMT compared to those on ETI (**Figure 2A**; NoHEMT: median 1553 ng/ml, n=9; ETI: median 930 ng/ml, n=30; p=0.0090). In contrast, MPO activity did not significantly differ between the two groups (**Figure 2B**; NoHEMT: median 4638 ng/ml, n=9; ETI: 932 ng/ml, n=25; p=0.2664). For both enzymes, there was a wide range of variation within groups. However, we noted that the significant difference in NE was driven by an obvious bimodal distribution of NE activity above or below 400 ng/ml among PwCF taking ETI, with 13 on 30 of the subjects (43%) falling below this threshold. Notably, only one of nine (11%) NoHEMT subjects also had sputum NE activity below the cutoff.

In summary, there were few clear differences between the sputum supernatant of NoHEMT and ETI groups, excepting NE activity which exhibited an obvious bimodal distribution within ETI subjects. Reasoning that the two sub-populations likely had major differences in disease that could influence their response to ETI, we decided to further investigate them.

Chapter 2: NE activity distinguishes two populations of PwCF undergoing ETI therapy

High or low NE activity distinguishes two populations of PwCF undergoing ETI therapy

In the context of low (NE^{Lo}) or high (NE^{Hi}) sputum NE activity among PwCF taking ETI, we sought to understand whether the two subgroups differed in demographics and overall disease (**Table 3**). Females were being significantly underrepresented in the NE^{Lo} subjects (1/13, 7.7%; Fisher's exact test p=0.0174). However, age, race, duration of ETI use, prior HEMT use, CFTR genotype, pathogen culture, insulin use, and BMI did not significantly differ between the two groups. All subjects in both groups were pancreatic enzyme insufficient.

We reinterrogated MPO, cytokines, and metabolites analysis. MPO activity was significantly increased in NE^{Hi} subjects compared to NE^{Lo} subjects (**Figure 3A**; p=0.0002). Similarly, 10 (of 18) cytokines and chemokines assessed were significantly different ($p<0.05$ and $q<0.05$, each). Three (CXCL5, CXCL10, and CXCL11; **Figure 3B-D**) were decreased in NE^{Hi} donors and eight (IFNγ, IL-1β, IL-6, IL-10, M-CSF, TNF-α, VEGF-A; **Figure 3E-K**) were increased. These data support an overall increase in inflammation and suppression of interferon responses in NE^{Hi} subjects, consistent with high neutrophil burden and rewiring of immune crosstalk.

We also interrogated metabolites in sputum, again subsetting for NE^{Hi} or NE^{Lo} subjects within PwCF taking ETI (**Figure 4**). High NE activity was associated with 107 (out of 380 total) metabolites (**Figure 4A**; $p<0.05$ and $q<0.05$), including amino acids, biogenic amines, phospholipids, nucleotides, and organic acids. Notably, all of the significant metabolites were increased in NE^{Hi}. Pathway analysis of the 107 selected metabolites revealed overrepresentation in five pathways ($p<0.05$, $q<0.05$): purine metabolism, branched chain amino acids, arginine metabolism, Phe/Trp/Tyr metabolism, and nicotinate metabolism (**Figure 4B**). In purine

metabolism, we noted a clear reactivity module connecting hypoxanthine and degradation products xanthine, xanthosine 5-phopshate, xanthosine, and allantoin (**Figure 4C**). We additionally noted metabolites consistent with NE and MPO activities among the significant results, including glycylleucine and essential amino acids (anticipated to be derived from protease activity) and dehydromethionine and methionine sulfoxide (anticipated to be from oxidative stress; **Figure 4C**). Overall, the metabolite results are consistent with broad neutrophil-driven pro-inflammatory metabolome disruption in the sputum supernatant of NE^{Hi} donors.

Worse lung function in the NE^{Hi} subset precedes and is maintained after ETI therapy

Lung function was significantly lower in NE^{Hi} subjects (**Table 3**; p=0.0117). Retrospective analysis of lung function history between NE^{Hi} and NE^{Lo} groups revealed clear separation before ETI introduction, with higher ppFEV1 in NE^{Lo} subjects (**Figure 5A**; p=0.0027 for NE activity subset effect by type III mixed effects model, and Sidak's multiple comparison test revealed $p<0.05$ difference between the two groups at each time point). However, the gap between the two groups narrowed by the third year post-ETI (**Figure 5A**). Lung function gains in the NE^{Hi} were overall not significantly different from those in the NE^{Lo} group, although NE^{Hi} exhibited more subjects with loss of lung function (**Figure 5B**). Taken together, these data indicate that NE^{Hi} subjects have worse lung function that predates undergoing ETI treatment and are not completely rescued by the use of modulator therapy.

Chapter 3: Discussion

We found that PwCF at our clinical center either taking ETI or no modulators exhibited few differences in assayed sputum molecules including neutrophil-derived effector proteins, pro-inflammatory mediators. The only significant difference in sputum was NE activity. However, this result was driven by a sub-population of individuals with low NE activity (NE^{Lo}). When we compared the NE^{Hi} and NE^{Lo} subpopulations, we detected broad differences in soluble mediators of inflammation (MPO, cytokines, chemokines) and metabolites (including purines, proteolytic products and oxidized amino acids previously associated with neutrophils[16]).

Neutrophilic inflammation is a major factor in the pathogenesis of CF lung disease.[17] CF airway neutrophils exhibit a phenotypic shift through exocytosis of primary and secondary granules and metabolic reprogramming.[18-20] Exocytosis of primary granules releases NE into the extracellular environment, causing degradation of lung connective tissue,[21,22] and MPO, which generates strong halogenating oxidants that can irreversibly modify biological molecules.[23-25] NE and MPO activities are well-established biomarkers for CF disease severity and have been associated with CF lung function, including in predictive roles.[25-29] The findings from our study highlight the relationship between NE activity and lung function in individuals with CF in the context of HEMT. The ETI therapy group demonstrated a consistent and significant negative correlation between NE activity and lung function, both before and after adjustment for confounders. This suggests that higher NE activity is associated with worse lung function even when accounting for other variables, underscoring the persistent impact of inflammation on pulmonary health despite the benefits of ETI therapy. CF lung inflammation is also associated with higher levels of

proinflammatory soluble immune mediators and potent neutrophil chemoattractant like IL-8, TNF-α, IL-1β and IL-6.[21,30,31] Previous work showed that ivacaftor has only little impact on sputum inflammatory markers in less than a year and modest effects thereafter.[32,33] ETI has shown potential to decrease sputum inflammatory markers like NE and IL-1β over time, in some cases within weeks, but not below disease control levels.[9-11] Looking at systemic mediators, introduction to ETI affects distinct chemokines associated with neutrophilic inflammation by lowering the plasma levels of CXCL5, CXCL11 and IL-8[10,34]. In our study, CF adults undergoing ETI therapy only exhibited differences in cytokine levels from NoHEMT controls if they had low NE activity. While this does not directly contradict the cited longitudinal studies, the results do suggest that inflammatory burden is not normalized by ETI and provides insight into the way advanced neutrophilic disease burden could predispose individuals to impaired beneficial responses to ETI.

Metabolomics is another method for corroborating important mechanisms of airways disease and inflammation in CF[35-37]. Prior publications have addressed the impact of lumacaftor-ivacaftor on airway and blood metabolites[38,39], but this is the first published study to the best of our knowledge that addresses the metabolomic effects of ETI on CF airways. We observed stark metabolomic differences between NE^{Hi} and NE^{Lo} subpopulations that indicated neutrophil involvement owing previously noted metabolic pathways impacted by neutrophils: purine degradation, proteolytic products, and oxidized amino acids.[16,25,40] These findings may enable biomarker development to predict ETI responsiveness, particularly for metabolites like methionine sulfoxide that sensitively reflect neutrophil exocytosis.[24,25] Our results also demonstrate the power of metabolomics to monitor drug responses, as we detected tezacaftor and ivacaftor in sputum. We did not detect elexacaftor in sputum, and it possible that this drug has a shorter half-life sputum supernatant than

ivacaftor and tezacaftor, but methodological limitations are another potential cause of this result, as the hydrophobic modulators eluted very early in our HILIC-MS method.

NE^{Hi} subjects had significantly worse lung function for multiple years prior to ETI introduction, and modulator therapy did not close the gap. Females were underrepresented in the NE^{Lo} group. The data suggest that high neutrophil burden makes CF lung disease resistant to rescue by ETI, and that females may be at higher risk of this. Greater decline in pulmonary function after exacerbations has been noted in CF females compared to males,[41] and pulmonary insufficiency is the main cause of death in CF.[42] Lung function in CF patients has overall improved, from 74.6% in 2007 to 85% in 2022, mostly due to the introduction of modulator therapy.[43] When looking at the trajectory of pulmonary function of the adults in CF in our study, the ppFEV1 of PwCF did generally improve in the years after starting ETI therapy. PwCF on ETI NE^{Hi} tended to be in the moderate (ppFEV1 40% to 69%) to mild (ppFEV1 70% to 89%) lung disease categories. On the other hand, NE^{Lo} PwCF tended to be in the mild to normal (ppFEV1 $\geq$ 90%) category. Being on ETI therapy did not negate this difference, although the gap between the sub-populations narrowed by the third year of therapy. In another study, children with more severe lung disease prior to ETI showed higher improvements in lung function than adults in this severity group, suggesting that the clinical response toward ETI might depend on age and lung disease severity prior to ETI initiation.[44] A longitudinal study found that while respiratory burden was alleviated by the use of ETI, PwCF with advanced lung disease are still plagued by respiratory symptoms.[45]

Our study presents several limitations. First, we had access to a limited volume inherent to sputum collection in adults in CF undergoing CFTR modulator therapy. To obtain the different types of

data presented in this work, we relied on sample-sparing/multiplexed sensitive assays, but nevertheless, sputum was insufficient for all assays (e.g., many samples were unavailable for metabolomics). Another limitation is that we had to decide empirically the value of NE activity level that determined the dichotomy of PwCF on ETI into two subpopulations. This cutoff would need to be refined through future studies. Regarding the mechanistic understanding on the impact of NE elastase activity, it would be interesting to (i) consider direct measurements of level of neutrophil in exudates and evaluate their phagocytic killing capacities; (ii) measure the level of other proteins involved in CF inflammation (like trypsin inhibitors). Finally, this was a single-center cross-sectional study, with a small number of subjects. We do not know whether the donors, particularly PwCF NE^{Lo}, saw greater or lesser changes in inflammatory mediators after ETI introduction. Future longitudinal analyses should factor NE activity-based subpopulations into analyses of modulator response to assess whether those with less initial burden also experience greater or lesser improvement in burden easing.

Conclusion

Since the introduction of ETI, many PwCF have seen pronounced improvement in clinical outcomes and life expectancy. However, modulator therapy is not available to all PwCF, highlighting the need for additional therapies[46-48]. Additionally, inflammatory mediators and disrupted metabolites remain high in modulator treated PwCF with established lung disease,[18,49] which our findings confirm and extend to those with more severe burden. Our study highlights the need for adjunctive neutrophil-targeting therapy for PwCF with remaining significant amounts of NE activity after introduction to ETI therapy.

Chapter 4: Materials and methods

Human subjects

Forty-one adults with CF provided spontaneously expectorated sputum at clinically stable visits (no current or recent pulmonary exacerbation). Subjects with CF were enrolled at Emory University and Children's Healthcare of Atlanta and informed consent was obtained from all participants prior to their involvement in the study, as part of the CF Biospecimen Repository (CFBR) protocol. All aspects of subject enrollment and sample collection were approved by the Emory University Institutional Review Board (IRB00042577). Subject demographics are summarized in **Table 1**.

Pulmonary function testing was assessed through measurement of percent predicted forced expiratory volume in one second (or ppFEV1), and data recovered from medical records along with demographic information. ppFEV1 was calculated from these data using the reference equations for pulmonary function as published by the Global Lung Function Initiative (GLI).[50]

Sputum processing

Expectorated sputum was homogenized and supernatant isolated as previously described.[51] In brief, 3 ml of PBS supplemented with 2.5 mM EDTA was added per g of sputum. The mixture was homogenized by passing through an 18G needle, up and down, 4 times per ml. The homogenized sputum homogenate was centrifuged at 800 *g* and 4 ˚C to yield cell-free supernatant, and this supernatant was centrifuged at 3000 *g* and 4 ˚C for 10 min to remove any bacteria that may be present. The resulting sputum supernatant was stored at -80˚C prior to assay.

Extracellular NE activity

Free NE activity was quantified in sputum using Förster resonance energy transfer (FRET) probe NEmo-1.[3] Forty µl of diluted sputum supernatant were added in polystyrene 96-well assay plates. The reaction was initiated by adding 5 µM NEmo-1 and reporter cleavage was recorded over time. A standard curve from known concentrations of purified human NE (Calbiochem, San Diego, CA, USA) was included in each assay. Values below the detection limit are reported as 0, as previously published.[3]

Cytokine and chemokine quantification

The concentration of immune mediators was calculated using a 20-plex chemiluminescent assay (U-PLEX; Meso Scale Diagnostics), according to the manufacturer's protocol. Sputum supernatants were analyzed for the following cytokines and chemokines: chemokine ligand (CCL) 2 (CCL2), CCL4, CXC motif chemokine ligand (CXCL) 5 (CXCL5), CXCL10, CXCL11, granulocyte colony-stimulating factor (G-CSF), G-macrophage CSF (GM-CSF), interferon gamma (IFN-γ), interleukin (IL)-1α (IL-1α), IL-1β, IL-6, IL-8, IL-10, IL-18, IL-1 receptor antagonist (IL-1RA), IL-22, IL-29, M-CSF, tumor necrosis factor α (TNF-α), and vascular endothelial growth factor A (VEGF-A). A value of half the lower limit of detection was imputed for samples determined to be below the lower limit, and conversely, twice the upper limit of detection was assigned for samples assayed over the upper limit (in both cases, before dilution factors were applied). Owing to large numbers of values below the lower limits of quantification (>50% in at least one experimental group), GM-CSF and IL-22 were ultimately excluded from analyses. Because IL-8 and IL-1RA concentrations were above limits of quantification in the Mesoscale assay, they were re-assayed using enzyme-linked immunoassay (ELISA) kits purchased

from RayBiotech, with standard calibration and HRP-based detection. In both cases, ELISA data are presented in lieu of Mesoscale.

Metabolomics

Metabolites in sputum were extracted and measured analogous to previously described methods.[36,52] In brief, 50 µl of sputum supernatant were mixed with 100 µl of 1:1 acetonitrile and methanol containing 12.5 µM d5-hippuric acid, vortexed for 5 s, and incubated on ice for 30 min. Samples were centrifuged at 20,000 *g* and 4 ˚C for 10 min to yield clear supernatant. One hundred µl of extract were transferred into a new tube and stored at -80 ˚C. A small, equal volume of each extract was pooled to make a global quality control. Data acquisition was conducted using a Vanquish Horizon liquid chromatography system coupled to a Q Exactive High Field Hybrid Orbitrap mass spectrometer (ThermoFisher). Two-and-a-half µl of extract per sample were injected for liquid chromatography with a 5 µm, 2.1 × 150 mm iHILIC-(P) Classic column (HILICON) pumped at 0.2 ml/min and 40 ˚C with a 15-min gradient of water containing 15 mM ammonium acetate, pH 9.4 (mobile phase A) or acetonitrile (mobile phase B). The gradient began at 10% A and progressed to 90% A, followed by a 2-min hold and 8-min re-equilibration of the column at 10% A. Column eluate was introduced to a heated electrospray ionization source held at 320 ˚C and +3.5 or -2.75 kV for positive and negative ionization modes, respectively, and an ion capillary temperature of 275 ˚C. The automatic gain control (AGC) was set to $1x10^6$ ions with a maximum injection time of 200 ms. Scan range was 67–1000 *m*/*z* and Orbitrap resolution was 120,000 full width at half maximum (FWHM). We used Top20 N for data-dependent MS/MS acquisition, with dynamic exclusion of 8.0 s, AGC of $1x10^5$, and maximum injection time of 25 ms. The data acquisition sequence was randomized before injection and the global quality control was injected

every 10 samples to determine measurement reproducibility. Data was analyzed using Compound Discoverer 3.3 and FreeStyle 1.7 (Thermo Fisher Scientific). mzCloud (mzcloud.org) and LipidSearch (ThermoFisher) were used for MS/MS identifications.

Statistical analysis

Analyses were conducted in GraphPad Prism (10.1.1), Microsoft Excel and SAS (9.4). We employed nonparametric statistics to describe central tendency (median, interquartile range), assess group differences (Mann-Whitney test) and establish measures of association (Fisher's exact test). Correlation analyses were conducted to examine the relationship between NE activity and lung function. Both unadjusted and adjusted models were used. The adjusted models included the following confounders: age, gender, level of MPO activity, and CFTR genotype. Metabolomic data were analyzed using Metaboanalyst (metaboanalyst.ca),[53] with $\log_{10}$ data transformation and autoscaling prior to analysis, and Wilcoxon rank-sum test instead of Mann-Whitney test (data were not paired). When conducting cytokine and metabolite analyses, owing to multiple variable testing within either assay, p-values were controlled using the Benjamini-Hochberg correction ($q<0.05$).

Table 1. Characteristics of PwCF, distributed by use of ETI therapy.

Variable	NoHEMT	ETI	p-value
N	9	32	
Age (years)	28 (23 - 35)	28 (25 - 33.5)	0.7762
Female (%)	44.4	34.4	0.7010[d]
Race (%)[a]			0.0850[d]
-White or Caucasian	87.5	90.6	
-Black or African American	0.0	9.4	
-Asian	12.5	0.0	
CFTR Genotype (%)			0.0600[d]
-F508del Homozygous	44.4	62.5	
-F508del Heterozygous	22.2	34.4	
-Other	33.4	3.1	
ppFEV1[b] (median, IQR)	62.9 (52.2 - 85.1)	75.5 (56.4 - 91.4)	0.4761
BMI[c] (median, IQR)	21.5 (19.2 - 23.9)	24.4 (21 - 26.2)	0.2202
ETI Duration (months)	0 (0 - 0)	14 (11 - 19)	-
Insulin (%)			0.6586[d]
-Yes	11.1	21.9	
-No	88.9	78.1	
Pancreatic sufficiency (%)			0.2195[d]
-Yes	11.1	0	
-No	88.9	100	

P-values are derived from Mann-Whitney test unless otherwise indicated. a. n=8, NoHEMT. b. n=31, ETI. c. n=7, NoHEMT, and n=23, ETI. d. Fisher's exact test. $P<0.05$*, $p<0.01$**, $p<0.001$***.

Abbreviations: ETI, elexacaftor/tezacaftor/ivacaftor; CFTR, CF transmembrane conductance regulator; ppFEV1, percent predicted forced expiratory volume in one second; IQR, interquartile range.

Table 2. Correlation between NE activity and lung function in the different groups of PwCF.

	Unadjusted model		**Adjusted model***	
	Correlation coefficient	p-value	Correlation coefficient	p-value
NoHEMT (N=9)	-0.471	0.201	0.699	0.189
On ETI therapy (N=30)	-0.436	0.018	-0.477	0.029

*Adjusted model includes age, gender, level of MPO activity, and CFTR genotype.

Table 3. Characteristics of PwCF on ETI, distributed by level of NE activity.

Variable	**NE^{Hi}**	**NE^{Lo}**	**p-value**
N	17	13	
Age (years)	28 (25 - 31)	28 (25 - 35)	0.7689
Female (%)	52.9	7.7	0.0174[c]
Race (%)			0.5645[c]
-White or Caucasian	94.2	84.6	
-Black or African American	5.8	15.4	
-Asian	0	0	
CFTR Genotype (%)			0.1925[c]
-F508del Homozygous	47.1	76.9	
-F508del Heterozygous	47.1	23.1	
-Other	5.8	0	
ppFEV1[a] (median, IQR)	61.9 (49.2 - 83.4)	90.2 (70.3 - 105.5)	0.0117
BMI[b] (median, IQR)	22.3 (20.2 - 25.7)	25.1 (24.4 - 26.4)	0.1130
ETI Duration (months)	13 (11 - 20)	15 (10 - 18)	0.9666
Insulin (%)			0.6725[c]
-Yes	23.5	15.4	
-No	76.5	84.6	
Pancreatic sufficiency (%)			-
-Yes	0	0	
-No	100	100	

Being NE^{Hi} means showing a level of neutrophil elastase activity >= 400 ng/mL, with the opposite being labeled NE^{Lo}. P-values are derived from Mann-Whitney test unless otherwise indicated. a. n=16, NE^{Hi}. b. n=11, NE^{Hi} and n=10, NE^{Lo}. c. Fisher's exact test. $P<0.05$*, $p<0.01$**, $p<0.001$***. Abbreviations: ETI, elexacaftor/tezacaftor/ivacaftor; CFTR, CF transmembrane conductance regulator; ppFEV1, percent predicted forced expiratory volume in one second; IQR, interquartile range.

Figure 1. PwCF taking ETI present similar level of cytokine burden than PwCF not taking any HEMT. Eighteen cytokines and chemokines were assayed using Mesoscale or ELISA assay. Statistical significance is derived from Mann-Whitney test, adjusted by the Benjamini-Hochberg correction for multiple testing. All data are presented as median with interquartile range.

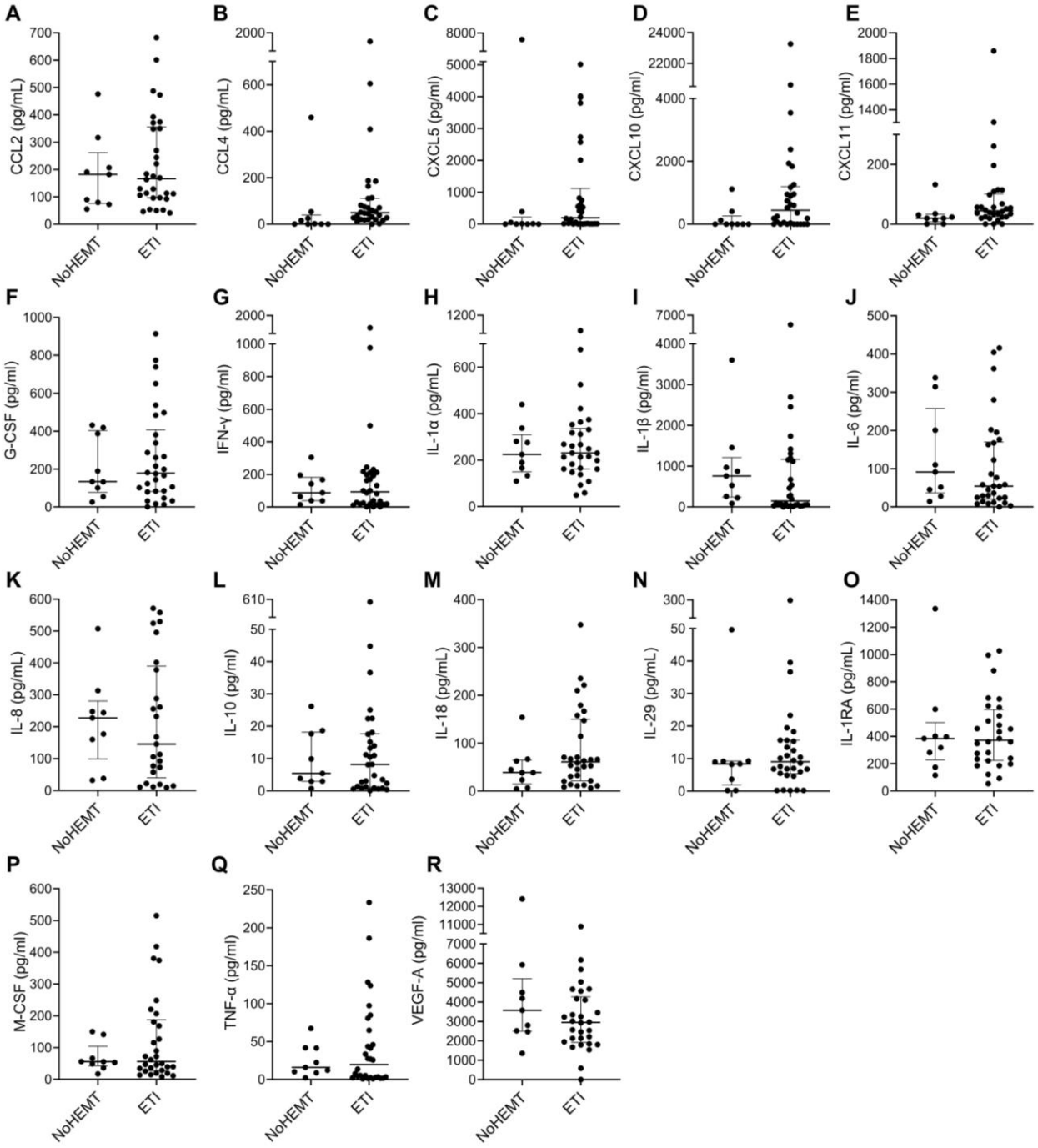


Figure 2. PwCF taking ETI had lower NE activity driven by an activity low sub-population. (A) NE activity in sputum supernatant was statistically significant owing to an activity low sub-population. The cutoff of 400 ng/ml activity, delineating NE^{Hi} and NE^{Lo} subpopulations, is marked with a dashed line. (B) MPO activity in sputum supernatant was not statistically significant between the two groups. Statistical significance is derived from Mann-Whitney test.

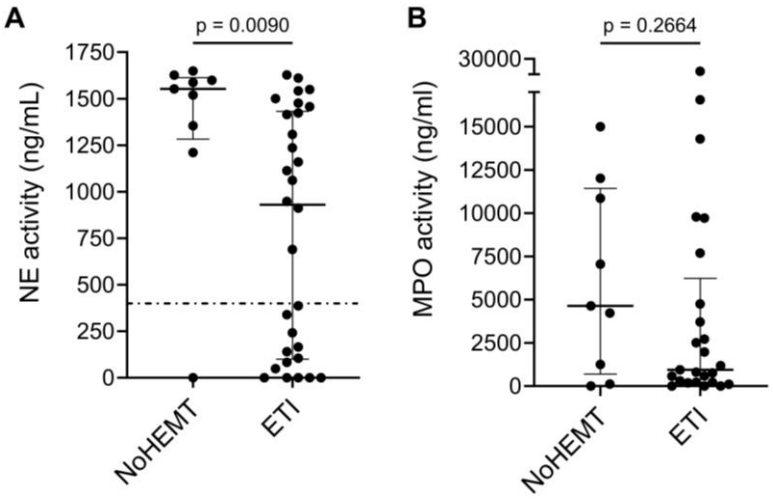


Figure 3. PwCF taking ETI with high NE activity have increased sputum MPO activity and pro-inflammatory cytokine burden. (A) MPO activity significantly differed between NE^{Hi} and NE^{Lo} subpopulations. (B-K) Ten cytokines and chemokines assayed using Mesoscale or ELISA assay that were significantly different between NE^{Hi} and NE^{Lo} subpopulations. Statistical significance is derived from Mann-Whitney test, adjusted by the Benjamini-Hochberg correction for multiple testing. All data are presented as median with interquartile range.

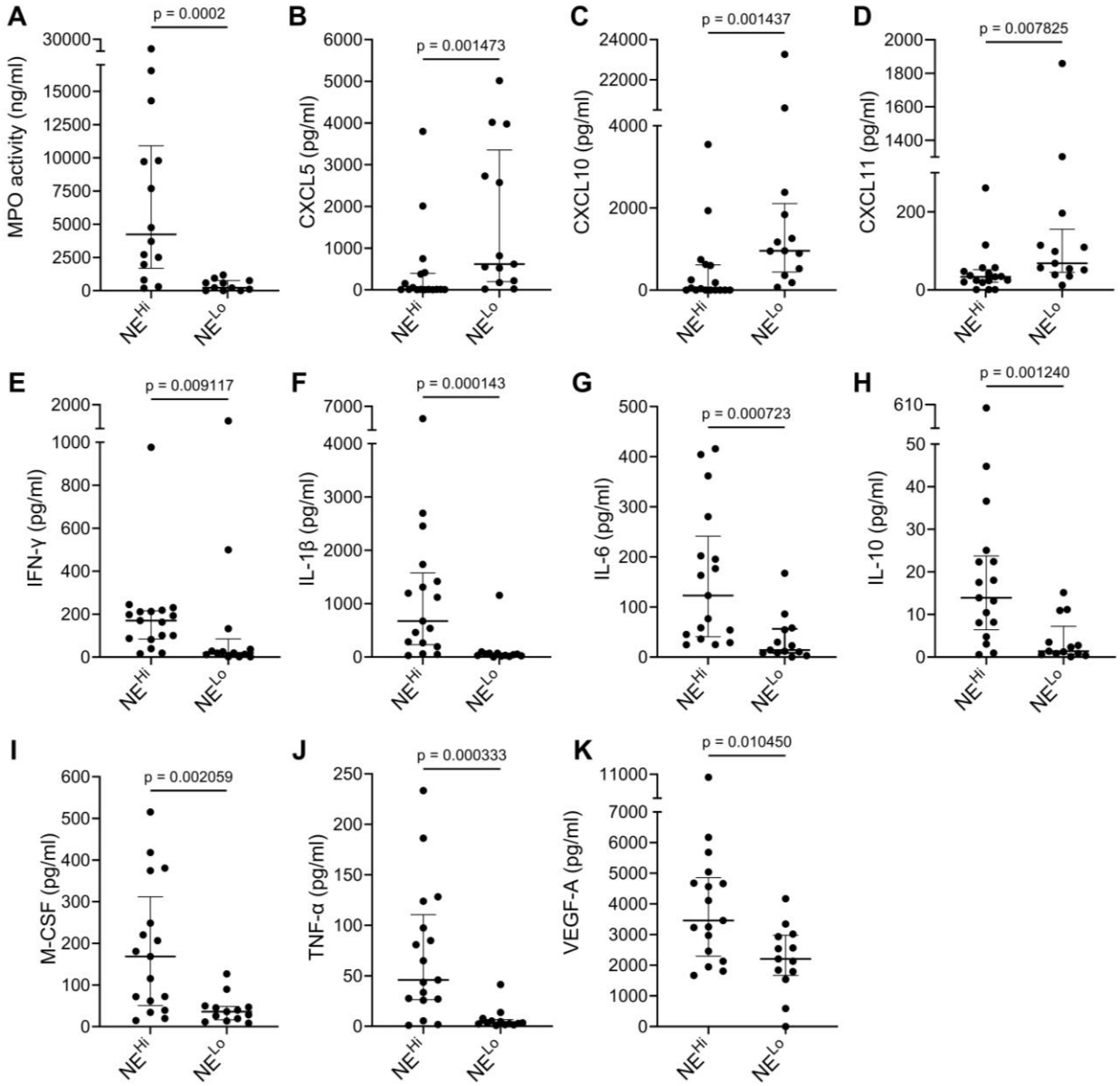


Figure 4. Metabolomics reveals broad differences between NE^{Hi} and NE^{Lo} donors with strong evidence of neutrophilic involvement. (A) Heatmap of 107 metabolites selected by Wilcoxon rank-sum test controlled by Benjamini-Hochberg correction of p-values. Data are clustered using Ward clustering with Euclidean distance. (B) Pathway analysis of 107 metabolites. Pathways that are $p<0.05$ and $q<0.05$ are named. (C) Select significant metabolites selected in (A) are shown grouped by major pathways associated with neutrophil activity, including purine degradation, peptides and essential amino acids, and oxidized amino acids. Individual data points are shown over bars of median and interquartile ranges.

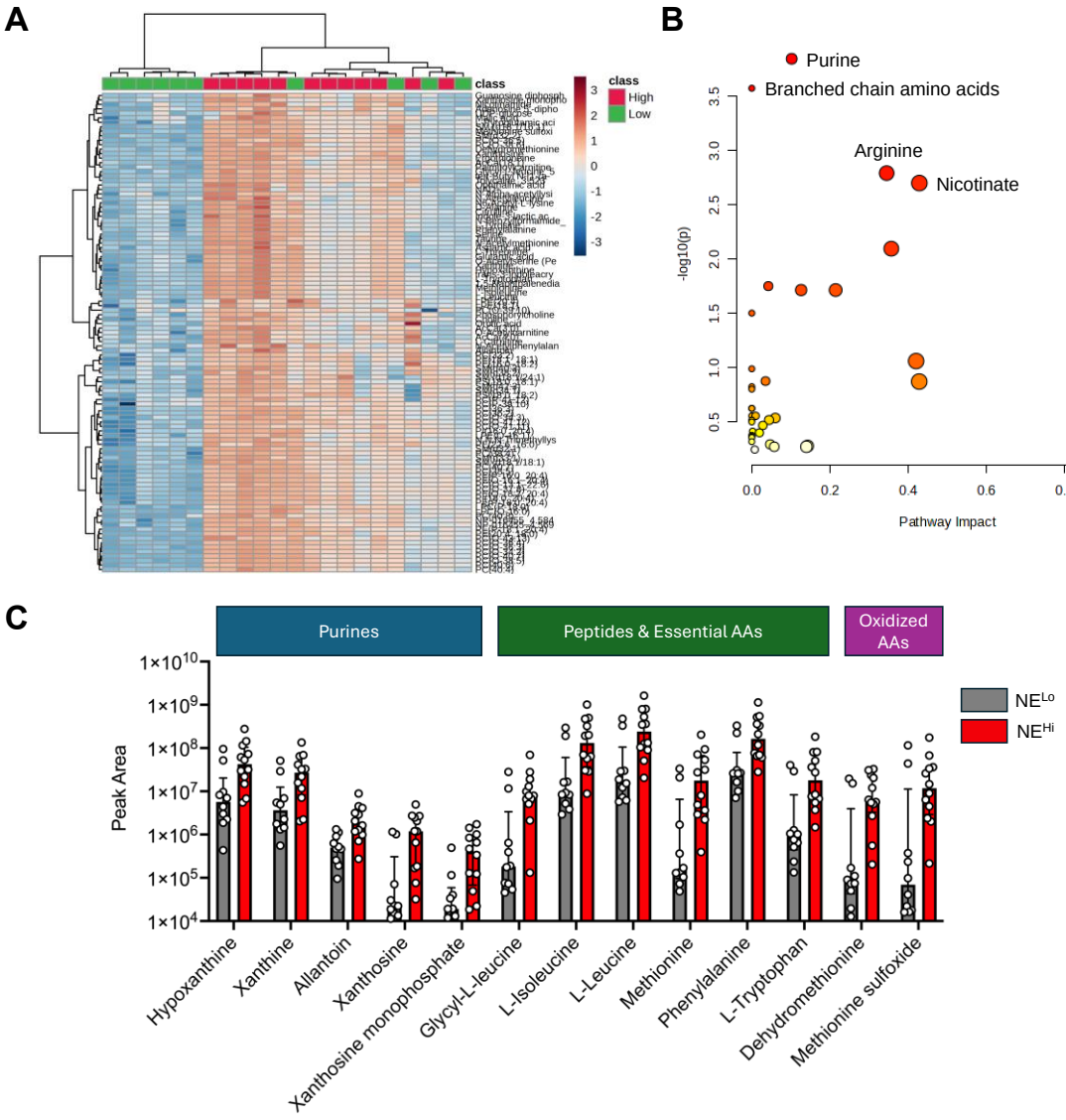
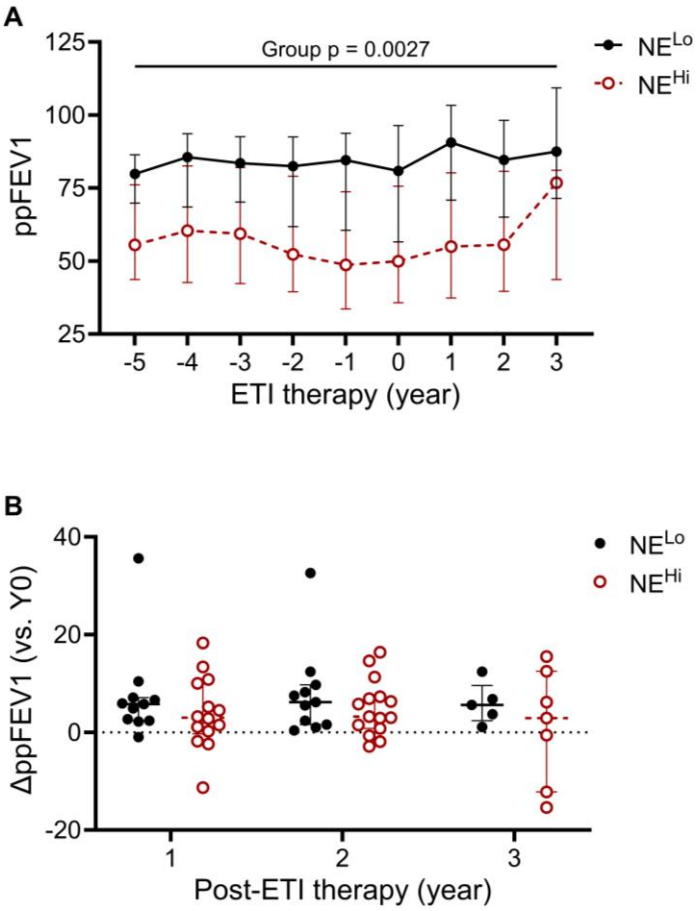


Figure 5. Forced expiratory volume in one second percent predicted (ppFEV1) is decreased in NE^{Hi} donors prior to the start of ETI and remains low. (A) Retrospective analysis of lung function was conducted from the medical record of sputum donors. A type III mixed effects model showed that the NE activity was significant (indicated on the graph) and Sidak multiple comparison test also showed significant differences of the individual time points. Data are median with interquartile range. (B) Change in ppFEV1 after the year of ETI introduction was tested for up to 3 years following start of therapy. No significant differences were observed.



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