Changes in Sputum Viscoelastic Properties and Airway Inflammation in Primary Ciliary Dyskinesia are Comparable to Cystic Fibrosis on Elexacaftor/Tezacaftor/Ivacaftor Therapy

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Online Supplementary Material

Supplementary methods

Study design and participants

Patients were eligible to participate if they had a confirmed PCD diagnosis according to the European Respiratory Society and American Thoracic Society guidelines [1, 2]. Patients with CF were included from the MODULATE-CF cohort and had at least one *F508del* allele and were aged 12 years and older as previously described [3]. CF patients from the MODULATE-CF cohort were matched with PCD patients for age, gender and the existence of matched sputum samples at baseline and at 3 months after initiation of treatment with ETI for paired analysis. Exclusion criteria were an acute respiratory infection or pulmonary exacerbation at the time of sputum collection [4]. Sputum rheology, inflammatory markers and proteomics were assessed in PCD patients that provided sputum at one of their routine visits to the outpatient clinic during the recruitment period, and in CF patients at baseline and 3 months after initiation of therapy with the approved dose of elexacaftor 200 mg and tezacaftor 100 mg every 24 hours in combination with ivacaftor 150 mg every 12 hours. For this subgroup of CF patients from the MODULATE-CF cohort, data on demographics, sputum rheology and inflammation markers were included in a previous study [3]. In the present study, additional inflammation markers such as DNA and MPO concentration and CatG and PR3 activity were determined and sputum proteomics was completely rerun and reanalyzed for comparison with PCD. Further, data of CF patients from MODULATE-CF were used for several additional analyses to assess the relationship between changes in viscoelastic properties and airway inflammation markers (supplementary figure S3 and S4), and the role of infection status and lung function impairment (supplementary tables S5 and S6).

Lung function

Spirometry was conducted and forced expiratory volume in one second (FEV1) was determined according to European Respiratory Society and American Thoracic Society

guidelines [5]. Percent predicted results were premised on equations of the global lung function initiative [6].

Sweat chloride concentration (SCC)

Sweat tests in patients with CF were conducted according to the German national diagnostic guideline [7] and the guidelines of the Clinical and Laboratory Standards Institute [8]. Sweat samples were collected after stimulation by pilocarpine iontophoresis with the Macroduct® system (Model 3700, Wescor, Logan UT, USA) and chloride concentration was determined in a minimum volume of 30 µl by using a chloridometer (KWM 20 Chloridometer, Kreienbaum, Langenfeld, Germany).

Sputum collection in healthy individuals

Collection of sputum samples from healthy controls was accomplished after inhalation of hypertonic saline (NaCl 3-6%) using a PARI boy classic and a LC sprint nebulizer (PARI GmbH, Starnberg, Germany). Before inhalation, the mouth was flushed with water to reduce saliva.

Sputum rheology

Sputum samples were immediately put on ice after expectoration or induction. Saliva and possible debris were removed by gentle aspiration with a pipette. Rheological measurements were subsequently conducted with a cone and plate rheometer (Kinexus Pro+, Netzsch GmbH, Selb, Germany) using a stainless-steel cone-plate geometry (cone-diameter 20 mm, cone-angle 1°). Samples were transferred onto the lower static plate of the rheometer with a non-electrostatic spatula. After sample loading and between sequences, temperature equilibration (5 min at 37°C) was conducted to ensure sufficient network relaxation. Each measurement comprised an amplitude sweep and a frequency sweep downwards. The amplitude sweep was performed at 1 Hz with a shear deformation γ between 0.01 - 100%. The frequency sweep was conducted at a shear deformation of 2% within a range from 10 to 0.1 Hz. The elastic modulus

(storage modulus, G') and viscous modulus (loss modulus, G″) were determined directly from the linear viscoelastic region of the amplitude sweep. The effective mesh size ξ of the mucin network was calculated using the following formula as previously described [9]:

$$\xi = \left(\frac{k_B \cdot T}{G}\right)^{\frac{1}{3}}$$

with k_B being the Boltzmann constant, T the absolute temperature and G the shear modulus, that was approximated from the G' value of the frequency sweep at 1 Hz.

Sputum inflammation markers and DNA concentration

Sputum samples were treated with 10% (v/v) sputolysin (Calbiochem, Darmstadt, Germany) to obtain single-cell solutions. In cell-free sputum supernatants concentrations of interleukin (IL)-1 β, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) were measured using cytometric bead array kits according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). Myeloperoxidase (MPO) concentrations were determined in cell-free sputum supernatants by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY, USA). Free NE activity was measured in cell-free sputum supernatants using the FRET reporter NEmo-1 (Sirius Fine Chemicals, Bremen, Germany). Free cathepsin G (CatG) and proteinase 3 (PR3) activity in cell-free sputum supernatants was determined using the fluorescence-quenching substrates Abz-Glu-Pro-Phe-Trp-Glu-Asp-Gln-EDDnp and Abz-Val-Ala-Asp-Nva-Arg-Asp-Arg-Gln-EDDnp (PeptaNova, Sandhausen, Germany), respectively. Kinetic assays were performed at 25°C using a fluorescence microplate reader (SpectraMax iD5, Molecular Devices, San Jose, CA, USA) and reporter cleavage was recorded over time (for NE: $\lambda_{Excitation}$ = 354 nm, $\lambda_{Emission\ Donor}$ = 400 nm, $\lambda_{Emission\ Acceptor}$ = 490 nm; for CatG and PR3: $\lambda_{Excitation}$ = 320 nm, $\lambda_{Emission}$ = 420 nm). Subsequently, neutrophil protease activity was determined by applying an enzyme standard curve as previously described [10-12]. DNA concentrations were determined in cell-

free sputum supernatants using the Quant-iT PicoGreen dsDNA assay kit according to the manufacturer's instructions (Invitrogen, Waltham, MA, USA).

Sputum proteomics

Sodium dodecyl sulfate (SDS) buffer (4% SDS, 100 mM Tris-HCl pH 8, 1 mM EDTA, 150 mM NaCl) was added to sputum samples in a 1:1 volume to volume ratio followed by an incubation at 95°C for 10 min to inactivate and solubilize proteins. Protein concentration was measured by a BCA assay and 100 µg protein were used for downstream analysis. Proteins were reduced and alkylated with 10 mM dithiothreitol and 40 mM chloroacetamide at 95°C for 10 min, followed by Benzonase® endonuclease (25U, Merck, Darmstadt, Germany) treatment for 15 min. Subsequently, a single-pot solid-phase-enhanced sample-preparation (SP3) protocol was applied for protein clean-up [13]. Protein containing beads were treated with 2 µg peptide-N-glycosidase F (NEB, Ipswich, MA, USA) in 50 mM ABC buffer for 1 hour at 37°C, followed by enzymatic digestion with trypsin (Promega, Madison, WI, USA) and lysyl endopeptidase (Fujifilm Wako Pure Chemical Corporation, Richmond, VA, USA) at a 1:50 enzyme:substrate ratio overnight at 37°C. The peptide containing supernatant was collected and desalted using C18 stage tips [14]. Peptide samples (2 µg) were subsequently measured on an EASY-nLC 1200 System coupled to an Orbitrap HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) running on data dependent acquisition (DDA) mode as previously described [15]. Raw data were analyzed using the MaxQuant software package (Ver. 2.0.3.0; Max Planck Institute of Biochemistry, Martinsried, Germany) and a decoy human UniProt database (2023-03) [16]. Variable modifications of N-terminal acetylation, deamidation (N, Q), oxidation (M) and fixed modification of carbamidomethyl cysteine were selected. For peptide and protein identification a false discovery rate (FDR) of 1% was chosen, and unique and razor peptides were considered for quantification. Further, "Match between runs" and label-free quantitation (LFQ) algorithm were applied. Data analysis was done in R (V 4.2.2). Data were filtered by removing reverse hits, proteins only identified by site and potential contaminants and proteins identified by at least two peptides or at least 5 MS/MS counts with an Andromeda

score above 20. Outlier patient samples, defined by number of proteins identified (<550) and principal component analysis, were excluded and proteins identified in at least 25% of the remaining patient samples were included in further analysis. LFQ intensities were log2-transformed and missing values were substituted by random values deduced from a normal distribution with a width of 0.3 and a down shift of 1.8. Moderated t-testing and moderated F testing were applied for statistical analysis [17]. *P*-values were adjusted using the Benjamini-Hochberg method with cutoff values of 0.05 or 0.01. Single sample gene set enrichment analysis (ssGSEA) was conducted using the ssGSEA2.0 package by Broad Institute [18, 19] and over representation analysis of proteins/cluster analysis was performed using the clusterProfiler package [20]. For both analyses only gene ontology (GO) terms of biological processes by the MsigSB [19] with a minimum size of 50 genes were considered. Filtering for at least 50 genes in the gene sets was conducted to remove gene sets that are not well represented within the data. Filtering for similarity between gene sets was not performed to prevent introduction of a bias by a chosen score and to prevent a further reduction of the list of gene sets. Proteomics of sputum samples from CF patients included in the MODULATE-CF cohort [3] was completely rerun and reanalyzed for comparison with PCD. Raw data and a table with all identified proteins, including *p* and adjusted *q* values, are available on the Proteomics Identifications Database PRIDE [21] (https://www.ebi.ac.uk/pride/; project accession number: PXD061751).

Magnetic resonance imaging

T1-weighted sequences before and after intravenous contrast, T2-weighted sequences, and first-pass four-dimensional (4D) perfusion imaging were acquired using a clinical 1.5T MR scanner (Magnetom Avanto or Magnetom Aera, Siemens, Erlangen, Germany). Images were assessed by an experienced thoracic radiologist (FD) for abnormalities in lung morphology and perfusion using a dedicated morpho-functional MRI score as previously described [22-24]. Perfusion studies were performed with intravenous administration of macrocyclic gadolinium-based contrast medium. The MRI morphology score comprises subscores for (1) bronchial wall abnormalities (wall thickening and/or bronchiectasis), (2) mucus plugging, (3) sacculations and/or abscesses, (4) consolidations, and (5) pleural reaction including effusion. The extent of these structural abnormalities as well as abnormal perfusion are rated in each lobe as 0 (no abnormality), 1 (<50% of the lobe involved), or 2 (≥50% of the lobe involved). The MRI global score results from the sum of the MRI morphology and MRI perfusion score. In this real-world study, MRI studies were limited to 35 PCD patients and 24 CF patients, in part due to the limited availability of MRI scan time in the clinical setting. MRI scans of CF patients were obtained as part of the MODULATE-CF study [25], but reanalyzed for comparison with PCD.

Statistics

Data of subgroup analyses are presented as group median and interquartile ranges or mean with standard deviation and were compared by Mann-Whitney test, Wilcoxon matched-pairs signed rank test, one-way ANOVA or Kruskal-Wallis test, as appropriate. The Benjamini-Hochberg method was used for multiple comparisons correction. Correlations analysis was performed using Spearman correlation. $P < 0.05$ was accepted to indicate statistical significance.

Supplementary references

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Supplementary tables

Supplementary table S1. List of genotypes of included patients with primary ciliary dyskinesia.

Predicted Ciliary Ultrastructure	Gene	First allele	Second allele
ODA	*DNAI1 (NM_012144.4)*	*Exon 3, c.138del*	*Exon 3, c.138del*
ODA	*DNAI1 (NM_012144.4*	*Exon 1, c.48+2dupT*	*Exon 10, c.817-2A>T*
ODA	*DNAI1 (NM_012144.4*	*Exon 1, c.48+2dupT*	*Exon 1, c.48+2dupT*
ODA	*DNAI2 (NM_023036.6)*	*Exon 4, c.346-3T>G*	*Exon 4, c.346-3T>G*
ODA	*DNAH5 (NM_001369.3)*	*Exon 7, c974dupA*	*Exon 27, c.4348C>T*
ODA	*DNAH5 (NM_001369.3)*	*Exon 54, c.8998C>T*	*Exon 63, c.10815delT*
ODA	*DNAH5 (NM_001369.3)*	*Exon 50, c.8440_8447del*	*Exon 74, c.12753T>G*
ODA	*DNAH5 (NM_001369.3)*	*Exon 27, c.4236 C>T*	*Exon 57, c.9637del*
ODA	*DNAH5 (NM_001369.3)*	*Exon 50, c.8440_8447del*	*Exon 25, c.4053+1G>T/Exon 25, c.3880G>A, Exon 42 c.6970A>G, Exon 43 c.7223 T>C*
ODA	*DNAH5 (NM_001369.3)*	*Exon 61, c.10384C>T*	*Exon 72, c.12430C>T*
ODA	*DNAH5 (NM_001369.3)*	*Exon 50, c.8440_8447del*	*Exon 63, c.10815del/Exon 42 c.6970A>G, Exon 43 c.7223 T>C*
ODA	*ODAD2 (NM_018076.5)*	*Exon 15, c.2147T>G*	*Exon 19, c. 2825*
ODA	*ODAD2 (NM_018076.5)*	*Exon 18, c.2675C>A*	*Exon 18, c.2675C>A*
ODA	*ODAD1 (NM_001364171.2)*	*Exon 4, c.337C>T*	*Exon 7, c.742G>A*
IDA	*DNAH1 (NM_015512.5)*	*Exon 32, c.5140A>T*	*Exon, 69, c.11072C>T*
ODA + IDA	*CCDC103 (NM_213607.2)*	*Exon 4, c.568_569dupAG*	*Exon 4, c.568_569dupAG*
ODA + IDA	*DNAAF1 (NM_178452.6)*	*Exon 5 c.1349dupC*	*Exon 5 c.1349dupC*
ODA + IDA	*DNAAF1 (NM_178452.6*	*Exon 5, c.683C>T*	*Exon 5, c.683C>T*
ODA + IDA	*DNAAF11 (NM_012472.6)*	*Exon 5, c.436G>C*	*Exon 5, c.436G>C*
ODA + IDA	*SPAG1* (*NM_003114.5)*	*Exon 16, c.2014C>T*	*Exon 1 and 2, Del. Ex 1-2*
ODA + IDA	*DNAAF6 (NM_173494.2)*	*Exon 5, c.266G>A (X-linked)*	*- - -*
MTD	*CCDC40 (NM_017950.4)*	*Exon 3, c.248delC*	*Exon 3, c.248delC*

MTD	*CCDC39 (NM_181426.2)*	*Exon 19, c.2596G>T*	*Exon 19, c.2596G>T*
MTD	*CCDC65 (NM_033124.5)*	*Exon 2, c.268 C>T*	*Exon 2, c.268C>T*
CPD	*RSPH3 (NM_031924)*	*Exon 1, c45.73del*	*Exon 1, c45.73del*
CPD	*RSPH4A (NM_001010892.3)*	*Exon 3, c.1105G>C*	*Exon 3, c.1105G>C*
CPD	*RSPH4A (NM_001010892.3)*	*Exon 3, c.1453C>T*	*Exon 3, c.1453C>T*
CPD	*RSPH4A (NM_001010892.3)*	*Exon 3, c1453C>T*	*Exon 4, c.1707del*
NU/CPD	*HYDIN (NM_001270974.2)*	*Exon 18, c.23494C>A*	*Exon 64, c.10816G>T*
NU	*DNAH11 (NM_001277115.2)*	*Exon 75, c.12363C>G*	*Exon 82, c.13531_*36del*
NU	*DNAH11 (NM_001277115.2)*	*Exon 26, c.4669C>T*	*Exon 70, c.11374-18A>G*
NU	*DNAH11 (NM_001277115.2)*	*Exon 26, c.4904_4905delAT*	*Exon 77, c.12899G>A/Exon 79, c.13181C>G*

Definitions of abbreviations of ultrastructure defects and genes: CCDC39 = coiled-coil domain-containing protein 39; CCDC40 = coiled-coil domain-containing protein 40; CCDC103 = coiled-coil domain containing protein 103; CPD = central pair defect; DNAAF1 = dynein arm assembly factor 1; DNAAF11 = dynein arm assembly factor 11; DNAI1 = dynein axonemal intermediate chain 1; DNAI2 = dynein axonemal intermediate chain 2; DNAH 5 = dynein arm heavy chain 5; DNAH1 = dynein arm heavy chain 1; DNAH11 = dynein arm heavy chain 11; IDA = inner dynein arm; MTD = microtubular disorganization; NU = (near) normal ultrastructure; ODA = outer dynein arm; ODAD 1 = outer dynein arm docking complex subunit 1; ODAD 2 = outer dynein arm docking complex subunit 2; RSPH3 = radial spoke head protein 3 homolog; RSPH4A = radial spoke head protein 4 homolog A; SPAG1 = sperm associated antigen 1

Supplementary table S2. List of cystic fibrosis transmembrane conductance regulator (*CFTR*) genotypes of included patients with cystic fibrosis.

First allele	Second allele	Number of patients
F508del	*F508del*	21
F508del	*N1303K*	3
F508del	*R347P*	3
F508del	*G542X*	2
F508del	*CFTRdele2,3*	1
F508del	*3302T>A*	1
F508del	*R1158X*	1
F508del	*W1282X*	1
F508del	*R709X*	1
F508del	*1525-1G>A*	1
F508del	*2991del32*	1
F508del	*p.Leu863fs/p.iie1027Thr*	1
F508del	*CFTRdele17a,17b*	1
F508del	*1078delT*	1
F508del	*R1066C*	1

Supplementary table S3. Maintenance therapy in patients with primary ciliary dyskinesia and patients with cystic fibrosis at baseline and after initiation of elexacaftor/tezacaftor/ivacaftor.

Clinical characteristic		PCD patients	CF patients at baseline	CF patients with 3 months ETI
Number of individuals		42	40	40
Inhaled hypertonic saline	n (%)	33 (78.6%)	29 (72.5%)	27 (67.5%)
	Change (%)	---	---	-2 (5.0%)
	P value	---	---	0.626
rhDNase	n (%)	---	24 (60.0%)	23 (57.5%)
	Change (%)	---	---	-1 (2.5%)
	P value	---	---	0.820
Inhaled antibiotics	n (%)	6 (14.3%)	32 (80.0%)	32 (80.0%)
	Change (%)	---	---	0
	P value	---	---	---
Oral azitromycin	n (%)	18 (42.9%)	19 (47.5%)	19 (47.5%)
	Change (%)	---	---	0
	P value	---	---	---
Other systemic antibiotics	n (%)	6 (14.3%)	12 (30.0%)	8 (18.3%)
	Change (%)	---	---	-4 (10.0%)
	P value	---	---	0.302

Definitions of abbreviations: CF = cystic fibrosis; ETI = elexacaftor/tezacaftor/ivacaftor; PCD = primary ciliary dyskinesia; rhDNase = human recombinant deoxyribonuclease.

Supplementary table S4. Lung magnetic resonance imaging scores for patients with primary ciliary dyskinesia (PCD) and patients with cystic fibrosis (CF) before and after initiation of elexacaftor/tezacaftor/ivacaftor (ETI) therapy. Perfusion studies were obtained in 30 of 35 patients with PCD, 20 of 24 patients with CF at baseline and 21 of 24 patients with CF on ETI.

		PCD	CF baseline	CF ETI
Number of individuals	n	35	24	24
MRI global score	Prevalence n (%)	29 (96.7)	21 (100)	21 (100)
	Mean (±SD)	20.6 (±8.1)	31.2 (±5.9)***	26.1 (±9.2)*#
MRI morphology score	Prevalence n (%)	34 (97.1)	24 (100)	24 (100)
	Mean (±SD)	14.6 (±6.4)	22.4 (±6.7)**	18.6 (±7.2)*
MRI perfusion score	Prevalence n (%)	29 (96.7)	20 (100)	21 (100)
	Mean (±SD)	5.6 (±2.2)	9.4 (±1.4)***	7.9 (±2.5)**#
Bronchiectasis/wall thickening subscore	Prevalence n (%)	34 (97.1)	24 (100)	24 (100)
	Mean (±SD)	7.1 (±2.4)	10.4 (±1.8)***	9.9 (±2.4)***
Mucus plugging subscore	Prevalence n (%)	33 (94.3)	24 (100)	20 (83.3)
	Mean (±SD)	3.9 (±2.4)	6.6 (±2.7)**	4.6 (±3.2)#
Abscesses/sacculations subscore	Prevalence n (%)	0 (0.0)	5 (20.8)	3 (12.5)
	Mean (±SD)	0.0 (±0.0)	0.5 (±1.1)*	0.4 (±1.1)
Consolidation subscore	Prevalence n (%)	25 (71.4)	18 (75.0)	11 (45.8)
	Mean (±SD)	1.5 (±1.3)	1.8 (±1.7)	0.9 (±1.2)
Special findings subscore	Prevalence n (%)	27 (77.1)	17 (70.8)	18 (75.0)
	Mean (±SD)	2.0 (±1.9)	2.8 (±2.6)	2.7 (±2.4)

Definitions of abbreviations: CF = cystic fibrosis; ETI = elexacaftor/tezacaftor/ivacaftor; MRI = magnetic resonance imaging; PCD = primary ciliary dyskinesia; SD = standard deviation.

*P < 0.05 compared with PCD; **P < 0.001 compared with PCD; ***P < 0.0001 compared with PCD; #P < 0.05 compared with CF baseline

Supplementary table S5. Subgroup analysis of patients with primary ciliary dyskinesia and patients with cystic fibrosis at baseline and after initiation of elexacaftor/tezacaftor/ivacaftor according to FEV_1 % predicted.

		PCD			CF baseline			CF ETI		
FEV_1 % predicted		<40%	40-70%	>70%	<40%	40-70%	>70%	<40%	40-70%	>70%
G‘ (Pa)	n	2	15	21	24	12	4	13	21	6
	Mean (±SD)	4.8 (±2.8)	7.4 (±7.1)##	10.1 (±8.2)	19.4 (±14.6)	29.6 (±30.5)	7.9 (±7.5)	12.0 (±11.0)	9.9 (±9.5)##	8.8 (±4.2)
G‘‘ (Pa)	n	2	15	21	24	12	4	13	21	6
	Mean (±SD)	1.9 (±0.9)	2.0 (±1.0)#	2.8 (±2.3)	4.5 (±3.3)	7.6 (±8.7)	2.2 (±1.9)*	3.1 (±2.9)	2.7 (±2.2)#	2.2 (±1.4)
DNA (µg/ml)	n	3	15	21	21	12	3	13	17	6
	Mean (±SD)	4.1 (±2.7)#	6.0 (±2.8)	2.8 (±2.6)*	26.1 (±24.1)	18.3 (±18.2)	7.6 (±8.0)	6.1 (±3.2)##	5.5 (±3.2)#	5.5 (±4.8)
MPO (ng/ml)	n	3	15	21	21	12	3	13	17	6
	Mean (±SD)	208.5 (±107.1)	165.7 (±95.1)##	133.7 (±86.1)	333.1 (±160.6)	334.3 (±155.4)	269.7 (±234.5)	140.8 (±110.7)##	166.2 (±101.3)##	150.4 (±114.5)
IL-1β (ng/ml)	n	3	15	22	24	12	4	13	21	6
	Mean (±SD)	0.5 (±0.4)	4.5 (±12.4)	0.4 (±0.5)	2.8 (±2.7)	1.3 (±1.3)	1.5 (±1.7)	1.3 (±1.9)	0.9 (±1.3)	1.0 (±1.5)
IL-8 (ng/ml)	n	3	15	22	24	12	4	13	21	6
	Mean (±SD)	66.7 (±53.0)	120.6 (±212.1)	45.6 (±73.9)	56.9 (±40.4)	37.0 (±26.8)	17.0 (±12.5)	36.3 (±31.4)	28.8 (±22.8)	25.6 (±27.3)
IL-6 (pg/ml)	n	3	15	22	24	12	4	13	21	6
	Mean (±SD)	15.7 (±18.1)	21.4 (±40.3)	31.3 (±67.7)§§	6.2 (±6.3)	6.8 (±7.2)	8.0 (±4.7)	22.7 (±21.1)#	41.8 (±98.2)	120.9 (±76.5)*†
TNF-α (pg/ml)	n	3	15	22	24	12	4	13	21	6
	Mean (±SD)	0.7 (±0.6)#	62.1 (±119.8)	18.4 (±57.3)§	124.7 (±221.9)	35.4 (±44.0)	11.7 (±16.0)	78.4 (±124.0)	30.9 (±44.6)	43.1 (±60.7)
Free NE activity (µg/ml)	n	3	15	22	24	12	4	13	21	6
	Mean (±SD)	42.6 (±41.4)	43.4 (±45.1)	16.9 (±17.9)#	96.1 (±76.5)	61.8 (±40.1)	23.3 (±21.0)	54.6 (±55.8)	33.1 (±35.9)#	17.1 (±20.9)
Free CatG activity (µg/ml)	n	3	15	22	24	12	4	13	20	6
	Mean (±SD)	32.6 (±56.3)	7.6 (±18.7)	3.7 (±17.0)**	4.7 (±4.9)	3.0 (±2.8)	0.6 (±0.3)	0.6 (±0.9)###	1.0 (±2.5)##	0.4 (±0.5)
	n	3	15	22	24	12	4	13	20	6

Free PR3 activity (µg/ml)	Mean (±SD)	4.3 (±7.1)	3.7 (±3.3)	1.6 (±5.1)*	11.6 (±8.3)	6.7 (±8.4)	3.0 (±2.9)	2.3 (±3.9)###	2.3 (±4.1)##	1.0 (±1.4)

Definitions of abbreviations: CatG = cathepsin G; CF = cystic fibrosis; DNA = deoxyribonucleic acid; ETI = elexacaftor/tezacaftor/ivacaftor; FEV1 = forced expiratory volume in one second; G' = elastic modulus; G'' = viscous modulus; IL = interleukin; MPO = myeloperoxidase; NE = neutrophil elastase; PCD = primary ciliary dyskinesia; PR3 = proteinase 3; SD = standard deviation; TNF-α = tumor necrosis factor α.

*P < 0.05 compared with $ppFEV_1$=40-70% within patient group; **P < 0.01 compared with $ppFEV_1$=40-70% within patient group; $^{\dagger}P$ < 0.05 compared with $ppFEV_1$<40% within patient group; $^{\#}P$ < 0.05 compared with CF baseline within $ppFEV_1$ group; $^{\#\#}P$ < 0.01 compared with CF baseline within $ppFEV_1$ group; $^{\#\#\#}P$ < 0.001 compared with CF baseline within $ppFEV_1$ group; $^{\S}P$ < 0.05 compared with CF ETI within $ppFEV_1$ group; $^{\S\S}P$ < 0.01 compared with CF ETI within $ppFEV_1$ group.

Supplementary table S6. Subgroup analysis of patients with primary ciliary dyskinesia and patients with cystic fibrosis at baseline and after initiation of elexacaftor/tezacaftor/ivacaftor according to microbial status.

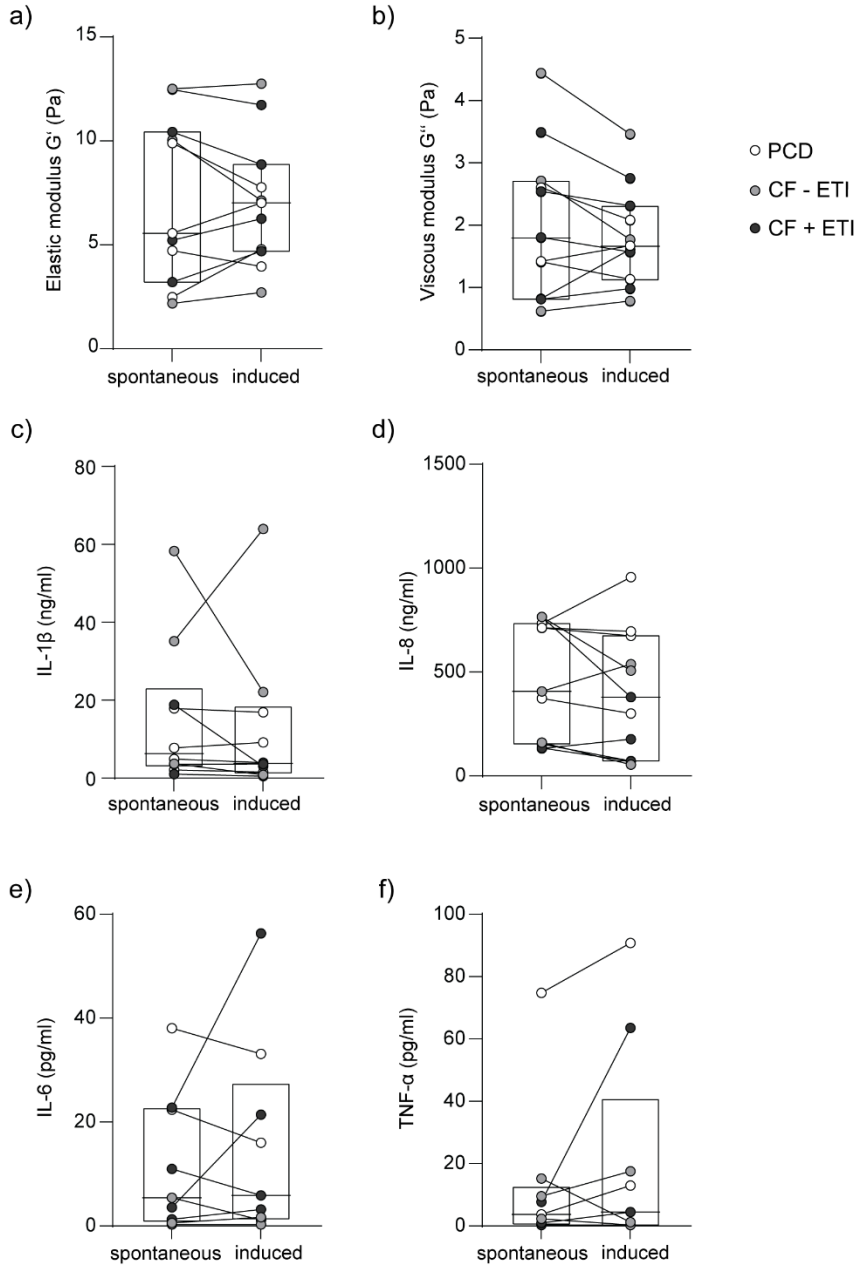
		PCD						**CF baseline**						**CF ETI**					
		H. influenzae		*S. aureus*		*P. aeruginosa*		*H. influenzae*		*S. aureus*		*P. aeruginosa*		*H. influenzae*		*S. aureus*		*P. aeruginosa*	
		-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
G‘ (Pa)	n	27	11	23	16	21	18	40	0	19	21	10	30	39	1	21	19	10	30
	Mean (±SD)	10.5 (±8.7)	5.7 (±2.9)	8.8 (±7.4)	9.7 (±8.3)	8.8 (±7.3)	9.6 (±8.4)	24.8 (±29.3)	-	20.9 (±25.4)	27.9 (±32.4)	16.9 (±12.8)	27.6 (±33.0)	13.1 (±16.3)	4.1 (±0.0)	3.2 (±2.8)	8.9 (±6.3)	7.7 (±4.5)	11.9 (±10.4)
	P value	0.251		0.899		0.967		---		0.690		0.438		---		0.002		0.523	
G‘‘ (Pa)	n	27	11	23	16	21	18	40	0	19	21	10	30	39	1	21	19	10	30
	Mean (±SD)	2.7 (±2.1)	1.8 (±0.8)	2.4 (±1.9)	2.5 (±1.8)	2.4 (±1.6)	2.6 (±2.1)	6.2 (±8.0)	-	5.4 (±7.4)	5.2 (±4.1)	4.0 (±2.8)	5.7 (±6.5)	3.4 (±4.0)	1.1 (±0.0)	3.2 (±2.8)	3.6 (±4.9)	2.0 (±1.4)	3.9 (±4.5)
	P value	0.450		0.927		0.906		---		0.769		0.527		---		0.931		0.263	
DNA (µg/ml)	n	29	11	25	16	22	19	36	0	16	20	8	28	35	1	20	16	8	28
	Mean (±SD)	4.3 (±3.1)	4.0 (±2.9)	3.4 (±3.0)	5.2 (±2.9)	3.0 (±2.8)	5.4 (±2.9)	22.0 (±21.7)	-	16.4 (±11.6)	26.4 (±26.8)	22.4 (±27.2)	21.8 (±20.5)	5.8 (±3.3)	0.2 (±0.0)	6.0 (±2.9)	5.3 (±4.0)	4.0 (±2.8)	6.2 (±3.5)
	P value	0.550		0.055		0.005		---		0.765		0.695		---		0.459		0.193	
MPO (ng/ml)	n	29	11	25	16	22	19	36	0	16	20	8	28	35	1	20	16	8	28
	Mean (±SD)	168.6 (±92.7)	111.7 (±73.1)	140.4 (±91.0)	168.1 (±89.0)	128.4 (±95.4)	177.6 (±77.8)	337.5 (±152.8)	-	307.6 (±173.2)	362.7 (±132.7)	343.6 (±174.4)	335.7 (±149.4)	163.4 (±100.3)	0.3 (±0.0)	160.6 (±104.7)	156.4 (±130.3)	175.1 (±129.1)	153.9 (±95.7)
	P value	0.090		0.217		0.045		---		0.589		0.954		---		0.882		0.384	
IL-1β (ng/ml)	n	29	12	26	16	23	19	40	0	19	21	10	30	39	1	21	19	10	30
	Mean (±SD)	0.7 (±1.1)	0.9 (±1.3)	0.6 (±0.9)	1.1 (±1.4)	0.63 (±1.0)	0.9 (±1.3)	2.2 (±2.3)	-	2.5 (±2.7)	1.9 (±1.9)	2.5 (±3.1)	2.1 (±2.1)	1.0 (±1.5)	0.0 (±0.0)	1.0 (±1.6)	1.0 (±1.4)	0.3 (±0.4)	1.2 (±1.7)
	P value	0.487		0.180		0.389		---		0.344		0.981		---		0.825		0.145	
IL-8 (ng/ml)	n	29	12	26	16	23	19	40	0	19	21	10	30	39	1	21	19	10	30
	Mean (±SD)	32.0 (±28.8)	36.2 (±36.1)	32.1 (±35.4)	47.0 (±37.5)	31.0 (±36.7)	45.0 (±35.5)	47.0 (±36.5)	-	51.2 (±32.3)	43.1 (±40.3)	48.1 (±39.3)	46.6 (±36.3)	31.5 (±26.0)	0.3 (±0.0)	31.0 (±26.8)	30.5 (±26.0)	23.8 (±18.0)	32.8 (±28.0)
	P value	0.903		0.206		0.157		---		0.282		0.975		---		0.979		0.483	
IL-6 (pg/ml)	n	29	12	26	16	23	19	40	0	19	21	10	30	39	1	21	19	10	30
	Mean (±SD)	15.7 (±33.6)	49.5 (±83.4)	34.7 (±74.6)	31.3 (±71.8)	49.0 (±92.5)	15.5 (±33.4)	6.5 (±6.4)	-	7.8 (±8.4)	5.4 (±3.7)	8.3 (±9.0)	6.0 (±5.5)	48.4 (±83.8)	9.6 (±0.0)	19.1 (±18.3)	78.8 (±112.0)	62.5 (±77.6)	43.1 (±85.1)
	P value	0.139		0.589		0.027		---		0.789		0.463		---		0.022		0.2907	
TNF-α (pg/ml)	n	29	12	26	16	23	19	40	0	19	21	10	30	39	1	21	19	10	30
	Mean (±SD)	29.9 (±101.7)	123.7 (±194.3)	32.7 (±92.1)	93.9 (±188.6)	45.4 (±101.3)	68.9 (±175.0)	84.9 (±175.1)	-	120.2 (±216.0)	53.0 (±124.5)	194.4 (±303.6)	53.1 (±103.1)	49.4 (±82.5)	0.4 (±0.0)	48.7 (±79.8)	47.6 (±86.2)	50.0 (±105.1)	47.6 (±75.9)
	P value	0.084		0.278		0.759		---		0.083		0.371		---		0.867		0.649	

Free NE activity (µg/ml)	n	29	12	26	16	23	19	40	0	19	21	10	30	39	1	21	19	10	30
	Mean (±SD)	25.6 (±24.5)	33.8 (±50.6)	25.6 (±31.4)	30.4 (±37.8)	24.8 (±38.6)	32.0 (±26.8)	78.6 (±66.2)	-	96.5 (±71.6)	62.4 (±57.9)	78.0 (±73.0)	78.8 (±65.4)	38.6 (±43.2)	2.4 (±0.0)	47.1 (±52.4)	27.3 (±27.1)	23.2 (±31.3)	41.9 (±45.4)
	P value	0.543		0.848		0.065		---		0.069		0.874		---		0.320		0.177	
Free CatG activity (µg/ml)	n	29	12	26	16	23	19	39	0	19	20	9	29	38	1	18	21	9	29
	Mean (±SD)	10.6 (±26.0)	0.8 (±1.8)	3.0 (±13.6)	15.0 (±30.4)	3.8 (±16.6)	12.1 (±27.0)	3.8 (±4.2)	-	3.5 (±3.1)	4.1 (±5.2)	3.3 (±4.1)	3.9 (±4.3)	0.8 (±1.9)	0.0 (±0.0)	1.0 (±2.5)	0.4 (±0.8)	0.4 (±0.8)	0.8 (±2.1)
	P value	0.2163		0.045		0.006		---		0.647		0.670		---		0.3431		0.966	
Free PR3 activity (µg/ml)	n	29	12	26	16	23	19	39	0	19	20	9	29	38	1	18	21	9	29
	Mean (±SD)	2.8 (±5.1)	2.4 (±3.1)	1.7 (±2.8)	4.2 (±6.3)	2.2 (±5.3)	3.2 (±3.6)	9.1 (±8.4)	-	9.2 (±9.1)	9.1 (±8.0)	8.9 (±9.2)	9.2 (±8.4)	2.1 (±3.7)	0.0 (±0.0)	2.1 (±3.8)	2.0 (±3.6)	1.7 (±3.4)	2.2 (±3.8)
	P value	0.871		0.084		0.097		---		0.813		0.635		---		0.631		0.955	

Definitions of abbreviations: CatG = cathepsin G; CF = cystic fibrosis; DNA = deoxyribonucleic acid; ETI = elexacaftor/tezacaftor/ivacaftor; G' = elastic modulus; G'' = viscous modulus; *H. influenzae* = *Haemophilus influenzae*; IL = interleukin; MPO = myeloperoxidase; NE = neutrophil elastase; *P. aeruginosa* = *Pseudomonas aeruginosa*; PCD = primary ciliary dyskinesia; PR3 = proteinase 3; *S. aureus* = *Staphylococcus aureus*; SD = standard deviation; TNF-α = tumor necrosis factor α

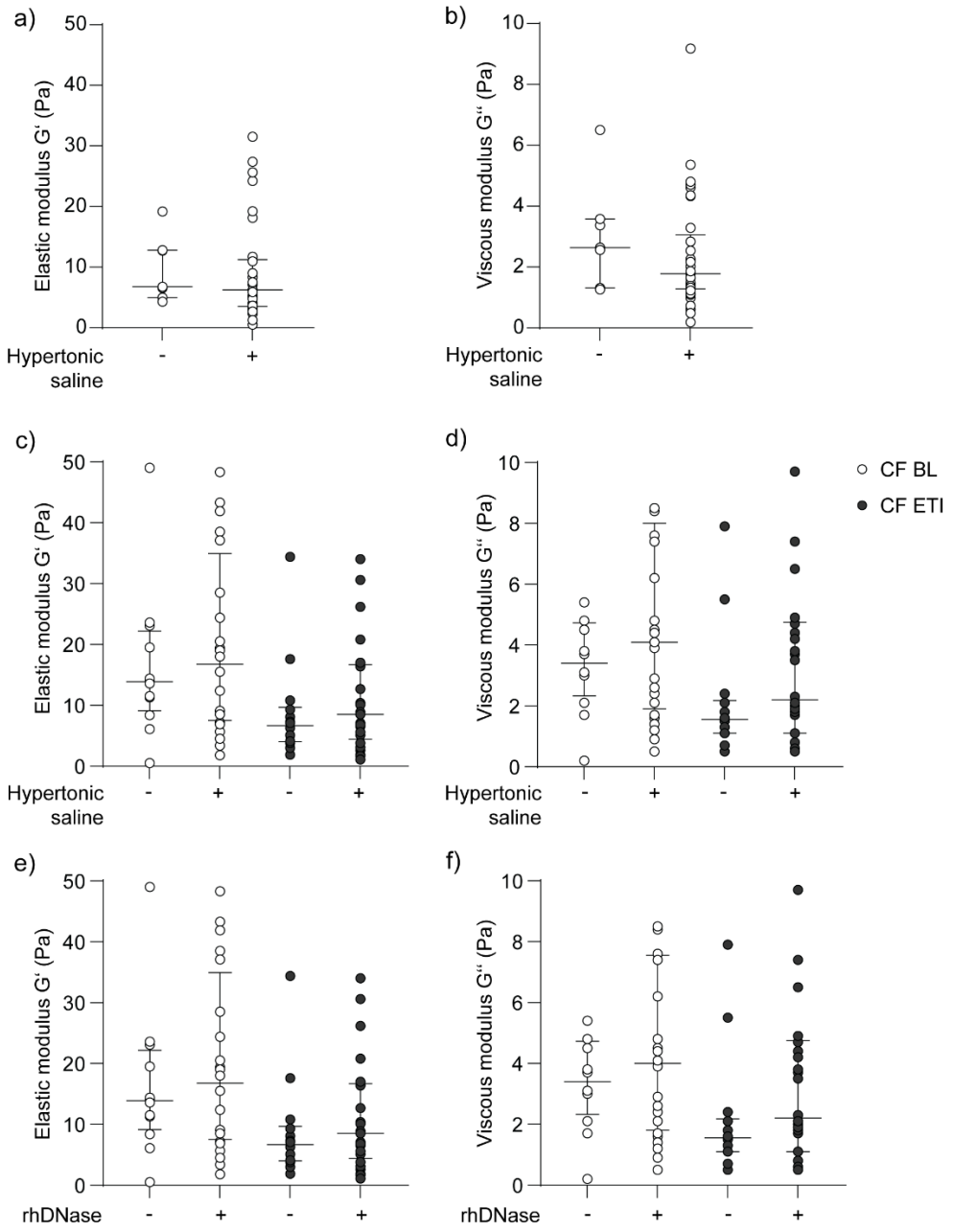
Supplementary figures

Supplementary figure S1



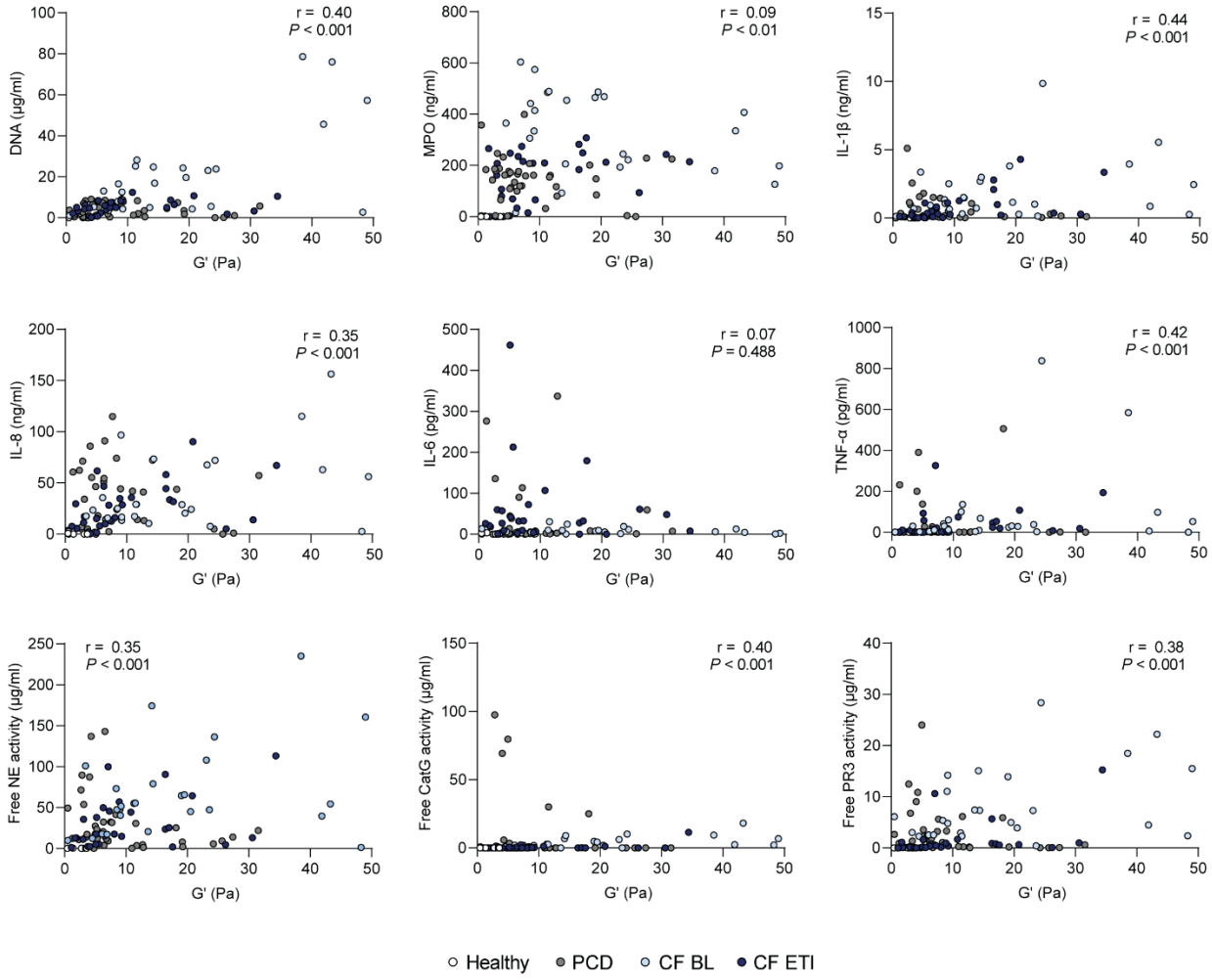
Supplementary figure S1. Comparison of viscoelastic properties and inflammation markers in spontaneously expectorated vs. induced sputum from patients with primary ciliary dyskinesia (PCD) (n = 4) and patients with cystic fibrosis (CF) without (- ETI; n = 3) or with elexacaftor/tezacaftor/ivacaftor (ETI) therapy (+ ETI; n = 4). Induced sputum was collected within 24 h after spontaneous sputum expectoration from the same patient. Elastic modulus G' (a), viscous modulus G'' (b). Interleukin (IL)-1β (c), IL-8 (d), IL-6 (e) and tumor necrosis factor (TNF)-α (f) levels in sputum supernatant. Boxes represent the 25th and 75th percentile with the group median (middle bar).

Supplementary figure S2



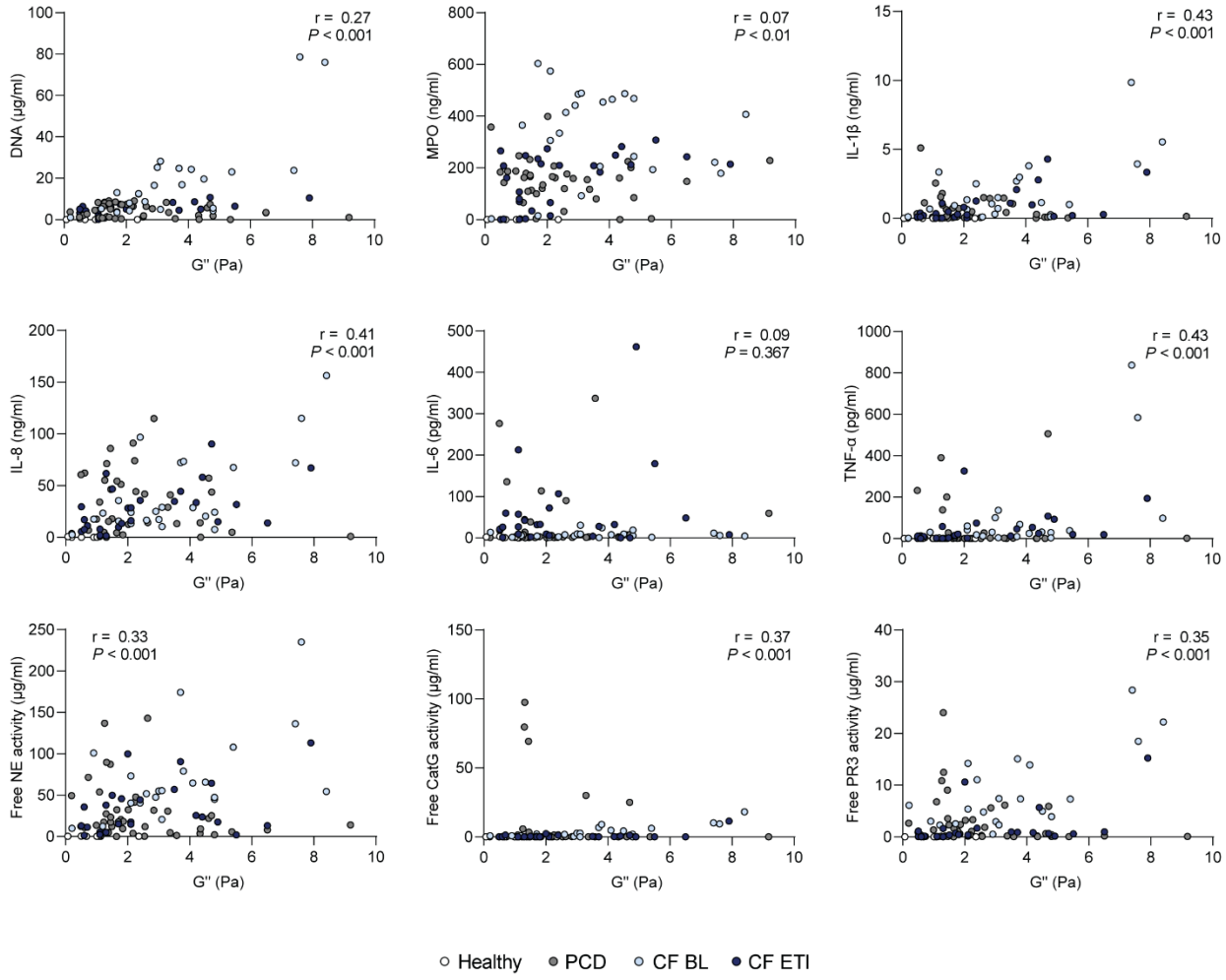
Supplementary figure S2. (a-b) Comparison of the elastic modulus G' (a) and viscous modulus G'' (b) of sputum from patients with primary ciliary dyskinesia (PCD) without (n = 7) or with (n = 32) inhaled hypertonic saline as maintenance therapy. (c-f) Sputum viscoelastic properties of patients with cystic fibrosis (CF) at baseline (BL) and at 3 months after initiation of elexacaftor/tezacaftor/ivacaftor (ETI) therapy without (BL: n = 11, ETI: n = 13) or with (BL: n = 29, ETI: n = 27) inhaled hypertonic saline (c-d) and without (BL: n = 16, ETI: n = 17) and with BL: n = 24, ETI: n = 23) inhalation therapy with rhDNase as maintenance therapy (e-f). Bars represent the group median, error bars represent the 25th and 75th percentile.

Supplementary figure S3



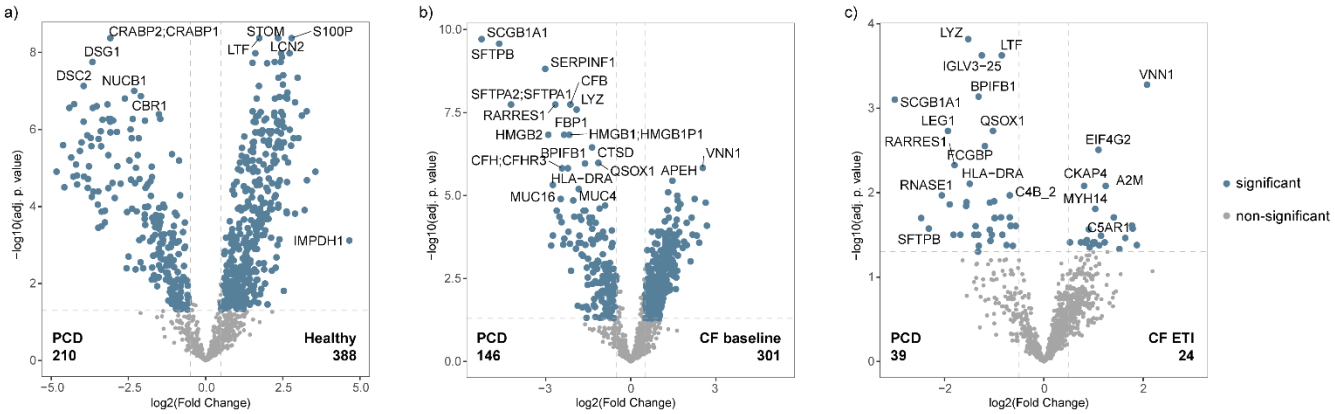
Supplementary figure S3. Relationship between the elastic modulus (G') and inflammation markers in sputum from the entire study population including healthy controls, patients with primary ciliary dyskinesia (PCD) and patients with cystic fibrosis (CF) at baseline (BL) and at 3 months after initiation of elexacaftor/tezacaftor/ivacaftor (ETI) therapy. Spearman correlation coefficients r and *P* values are provided for each correlation. Definitions of abbreviations: CatG = cathepsin G; DNA = deoxyribonucleic acid; IL = interleukin; MPO = myeloperoxidase; NE = neutrophil elastase; PR3 = proteinase 3; TNF-α = tumor necrosis factor α.

Supplementary figure S4



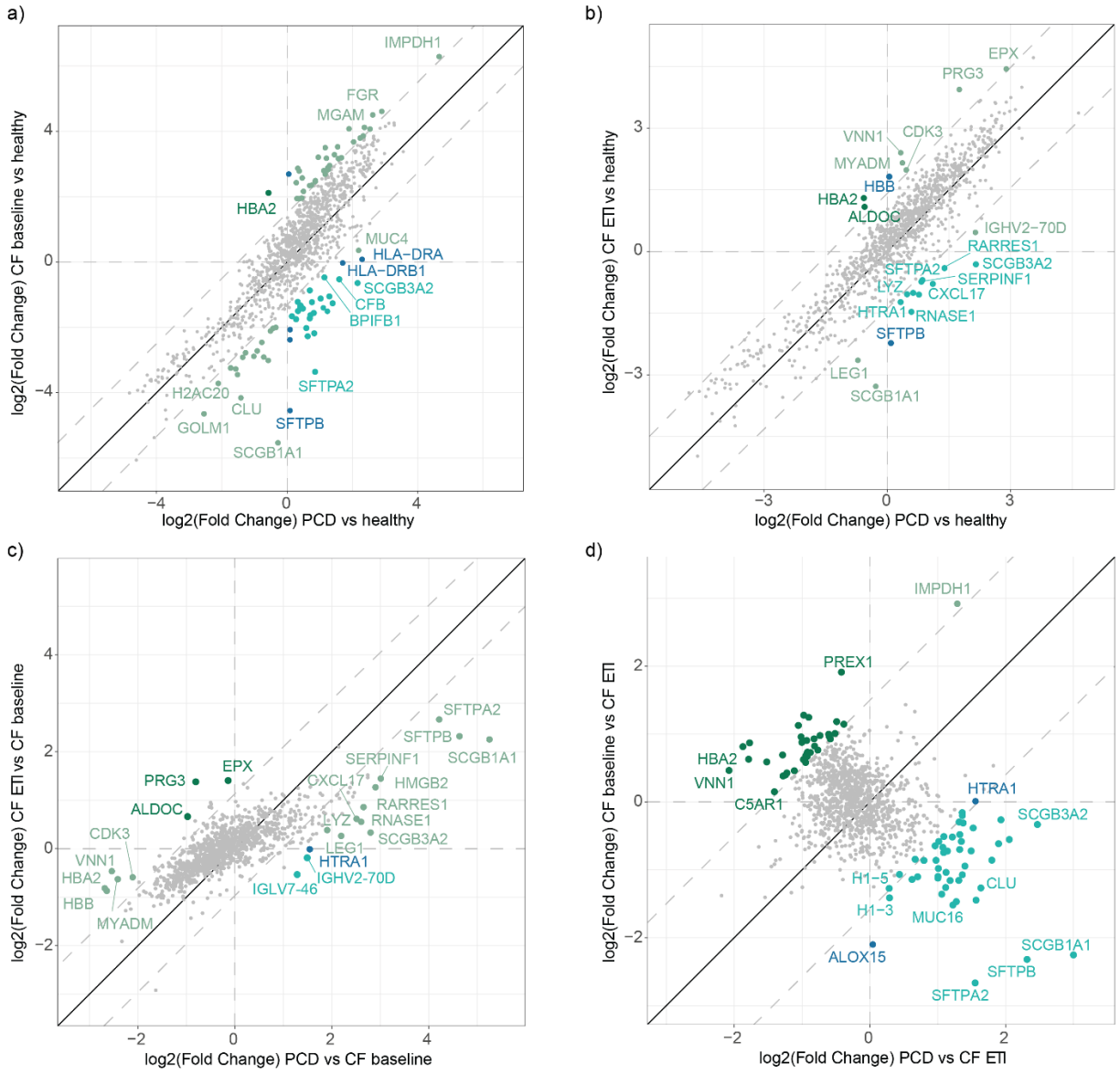
Supplementary figure S4. Relationship between the viscous modulus (G'') and inflammation markers in sputum from the entire study population including healthy controls, patients with primary ciliary dyskinesia (PCD) and patients with cystic fibrosis (CF) at baseline (BL) and at 3 months after initiation of elexacaftor/tezacaftor/ivacaftor (ETI) therapy. Spearman correlation coefficients r and *P* values are provided for each correlation. Definitions of abbreviations: CatG = cathepsin G; DNA = deoxyribonucleic acid; IL = interleukin; MPO = myeloperoxidase; NE = neutrophil elastase; PR3 = proteinase 3; TNF-α = tumor necrosis factor α.

Supplementary figure S5



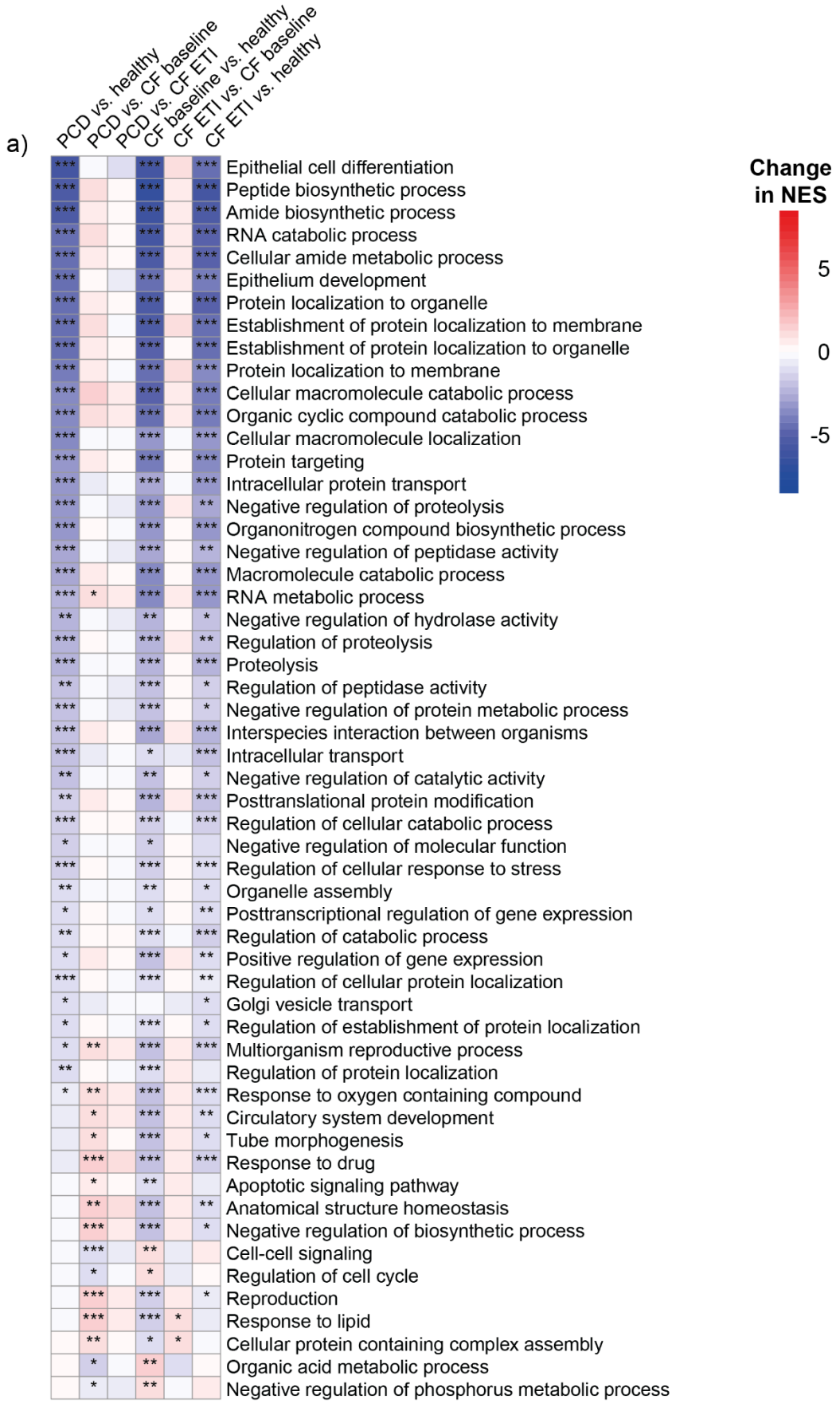
Supplementary figure S5. (a-c) Volcano plots demonstrating comparisons of protein expression in sputum from patients with primary ciliary dyskinesia (PCD) versus healthy individuals (598 differential proteins) (a), PCD patients versus patients with cystic fibrosis (CF) before elexacaftor/tezacaftor/ivacaftor (ETI) initiation (447 differential proteins) (b) and PCD patients versus CF patients at 3 months on ETI (63 differential proteins) (c). Moderated t-testing was used for statistical analysis and *P* values were adjusted by Benjamini-Hochberg correction with a cut-off value of 0.05 and a fold change cut-off of 0.5.

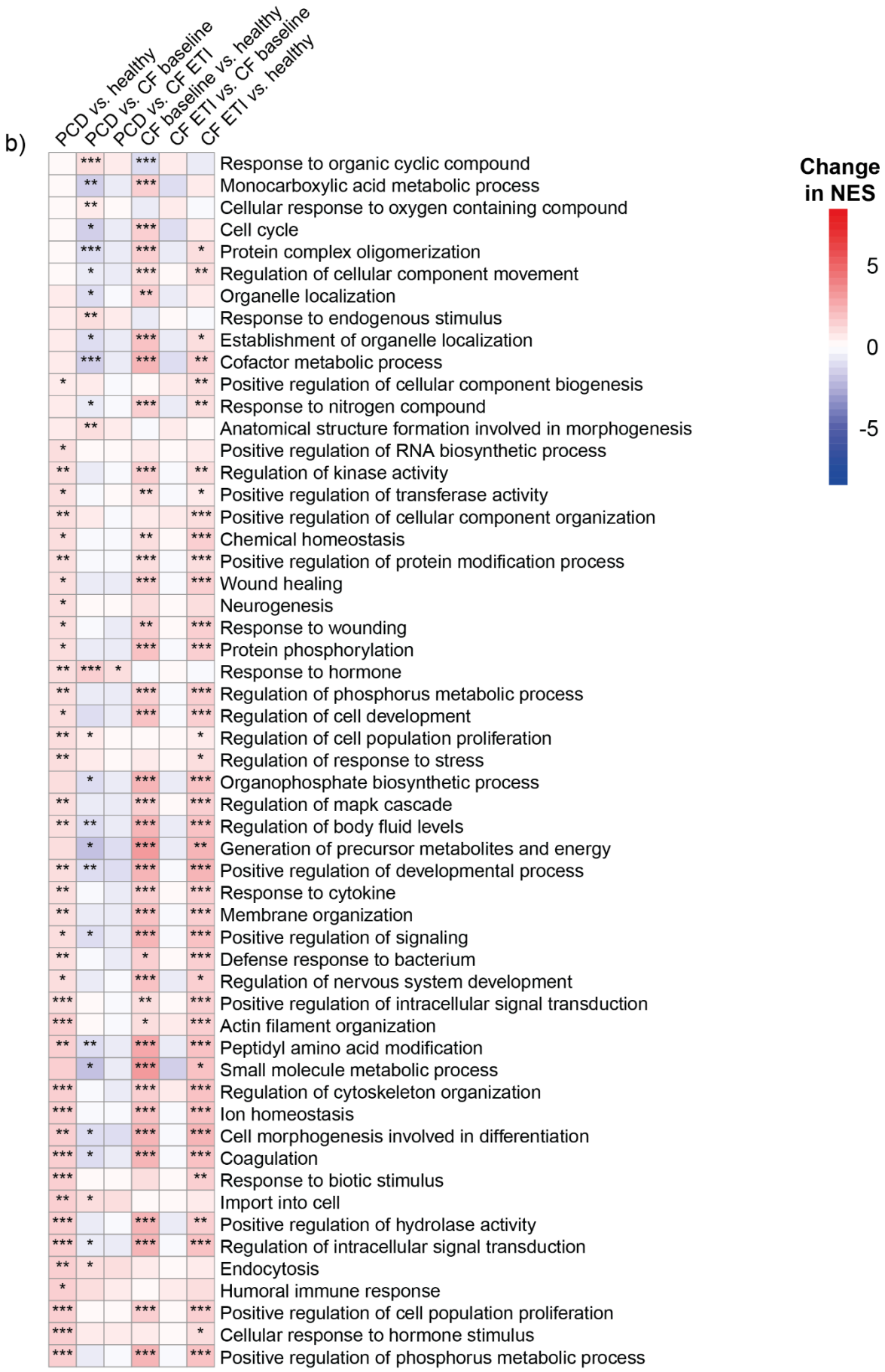
Supplementary figure S6



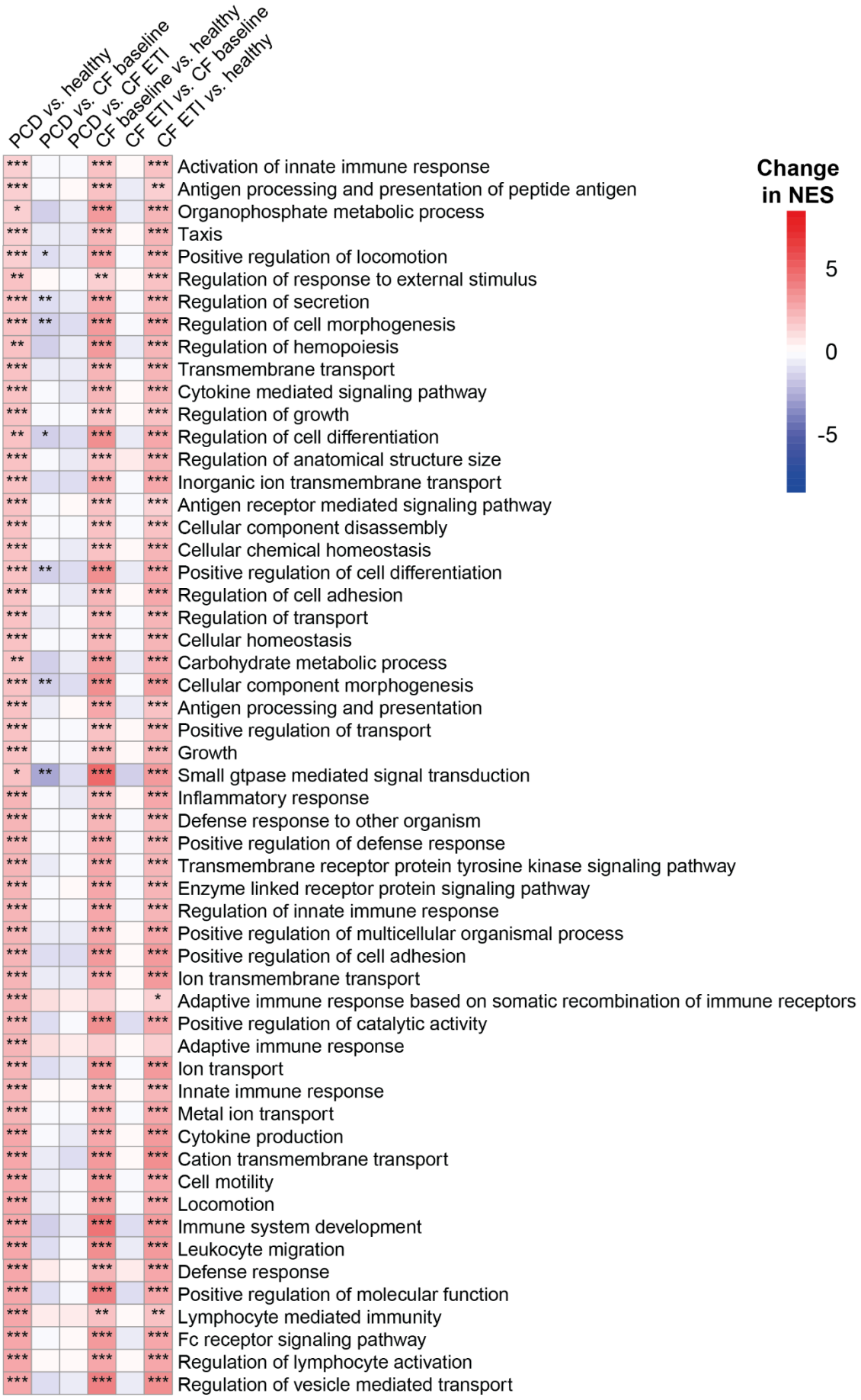
Supplementary figure S6. (a-d) Log2-fold changes of protein expression showing multiple comparisons of patients with primary ciliary dyskinesia (PCD) with patients with cystic fibrosis (CF) and healthy controls. Log2-fold protein expression was compared in PCD and CF patients before elexacaftor/tezacaftor/ivacaftor (ETI) treatment versus healthy individuals (a), in PCD and CF patients on 3 months ETI versus healthy individuals (b), in PCD patients and CF patients on 3 months ETI versus CF prior to ETI (c) and in PCD patients and CF patients at baseline versus CF patients at 3 months on ETI (d). Light green-colored proteins develop into the same direction, while certain proteins change only in one group (blue) or into opposite directions (dark green, cyan). A fold change cut-off value of 1.5 was chosen.

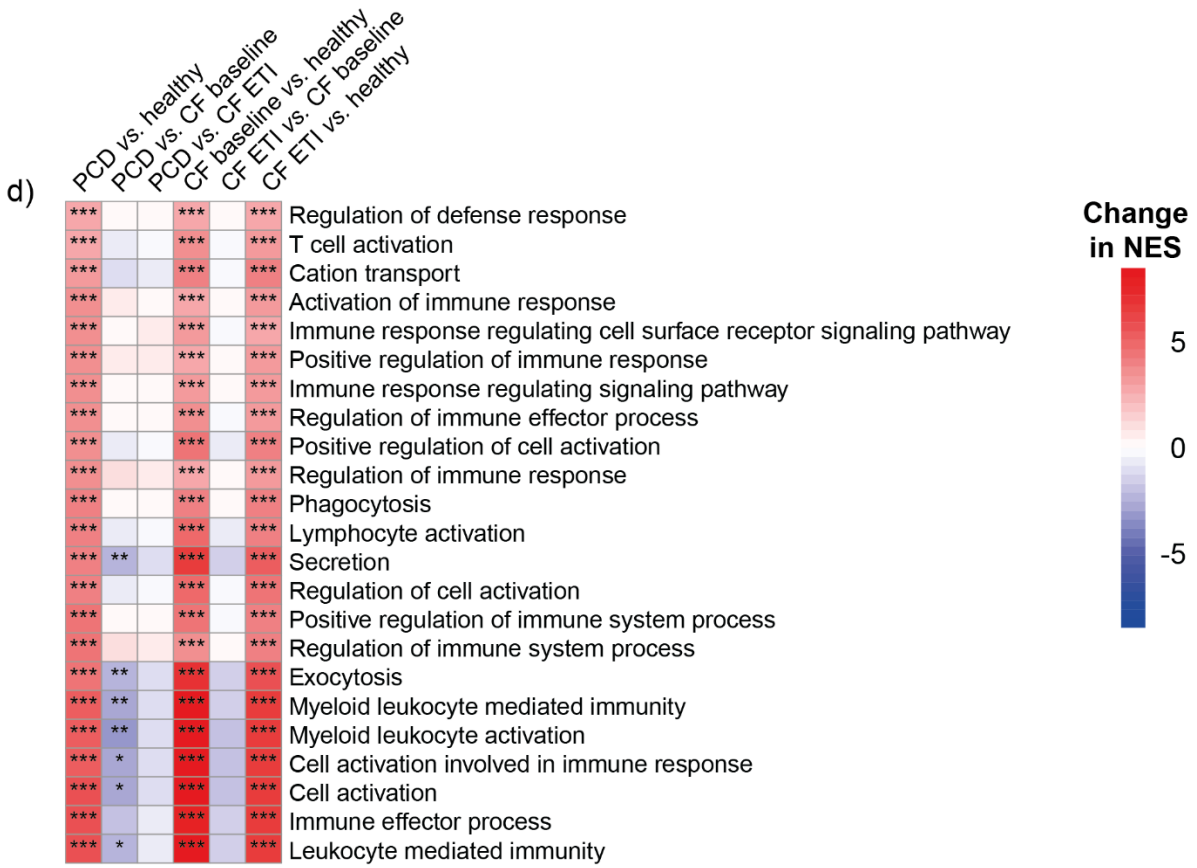
Supplementary figure S7




b)
PCD vs. healthy
PCD vs. CF baseline
PCD vs. CF ETI
CF baseline vs. healthy
CF ETI vs. CF baseline
CF ETI vs. healthy
Response to organic cyclic compound
Monocarboxylic acid metabolic process
Cellular response to oxygen containing compound
Cell cycle
Protein complex oligomerization
Regulation of cellular component movement
Organelle localization
Response to endogenous stimulus
Establishment of organelle localization
Cofactor metabolic process
Positive regulation of cellular component biogenesis
Response to nitrogen compound
Anatomical structure formation involved in morphogenesis
Positive regulation of RNA biosynthetic process
Regulation of kinase activity
Positive regulation of transferase activity
Positive regulation of cellular component organization
Chemical homeostasis
Positive regulation of protein modification process
Wound healing
Neurogenesis
Response to wounding
Protein phosphorylation
Response to hormone
Regulation of phosphorus metabolic process
Regulation of cell development
Regulation of cell population proliferation
Regulation of response to stress
Organophosphate biosynthetic process
Regulation of mapk cascade
Regulation of body fluid levels
Generation of precursor metabolites and energy
Positive regulation of developmental process
Response to cytokine
Membrane organization
Positive regulation of signaling
Defense response to bacterium
Regulation of nervous system development
Positive regulation of intracellular signal transduction
Actin filament organization
Peptidyl amino acid modification
Small molecule metabolic process
Regulation of cytoskeleton organization
Ion homeostasis
Cell morphogenesis involved in differentiation
Coagulation
Response to biotic stimulus
Import into cell
Positive regulation of hydrolase activity
Regulation of intracellular signal transduction
Endocytosis
Humoral immune response
Positive regulation of cell population proliferation
Cellular response to hormone stimulus
Positive regulation of phosphorus metabolic process
Change in NES
5
0
-5

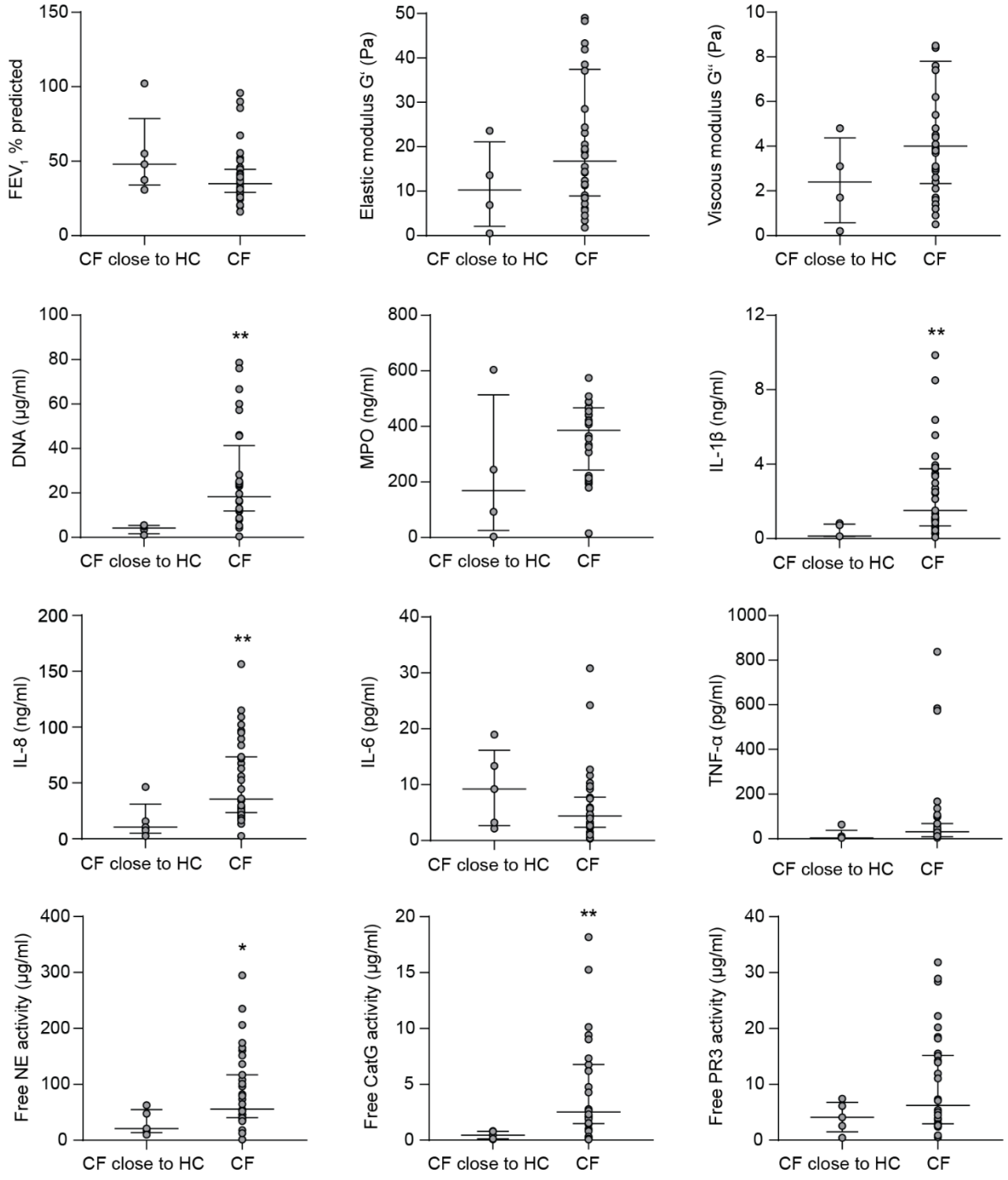
c)


PCD vs. healthy
PCD vs. CF baseline
PCD vs. CF ETI
CF baseline vs. healthy
CF ETI vs. CF baseline
CF ETI vs. healthy
Change in NES
5
0
-5
Activation of innate immune response
Antigen processing and presentation of peptide antigen
Organophosphate metabolic process
Taxis
Positive regulation of locomotion
Regulation of response to external stimulus
Regulation of secretion
Regulation of cell morphogenesis
Regulation of hemopoiesis
Transmembrane transport
Cytokine mediated signaling pathway
Regulation of growth
Regulation of cell differentiation
Regulation of anatomical structure size
Inorganic ion transmembrane transport
Antigen receptor mediated signaling pathway
Cellular component disassembly
Cellular chemical homeostasis
Positive regulation of cell differentiation
Regulation of cell adhesion
Regulation of transport
Cellular homeostasis
Carbohydrate metabolic process
Cellular component morphogenesis
Antigen processing and presentation
Positive regulation of transport
Growth
Small gtpase mediated signal transduction
Inflammatory response
Defense response to other organism
Positive regulation of defense response
Transmembrane receptor protein tyrosine kinase signaling pathway
Enzyme linked receptor protein signaling pathway
Regulation of innate immune response
Positive regulation of multicellular organismal process
Positive regulation of cell adhesion
Ion transmembrane transport
Adaptive immune response based on somatic recombination of immune receptors
Positive regulation of catalytic activity
Adaptive immune response
Ion transport
Innate immune response
Metal ion transport
Cytokine production
Cation transmembrane transport
Cell motility
Locomotion
Immune system development
Leukocyte migration
Defense response
Positive regulation of molecular function
Lymphocyte mediated immunity
Fc receptor signaling pathway
Regulation of lymphocyte activation
Regulation of vesicle mediated transport



Supplementary figure S7. (a-d) Heatmaps indicating enriched gene ontology (GO) terms of biological processes, ordered by change in normalized enrichment score (NES) (FDR < 0.01), in sputum of patients with primary ciliary dyskinesia (PCD) compared to healthy controls (first column), to patients with cystic fibrosis (CF) at baseline (second column) and to CF patients at 3 months after initiation of elexacaftor/tezacaftor/ivacaftor (ETI) (third column). In addition, GO terms were compared between patients with CF at baseline and healthy controls (fourth column), CF patients on ETI and at baseline (fifth column), and CF patients on ETI were compared to healthy controls (sixth column). Statistical significances are indicated as **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Supplementary figure S8



Supplementary figure S8. Comparison of indicated parameters between patients with cystic fibrosis (CF) at baseline that exhibit a proteomic signature more closely to healthy controls (n = 5) and the remaining CF patients (n = 35). Bars represent the group median, error bars represent the 25th and 75th percentile. *P < 0.05 and **P < 0.01. Definitions of abbreviations: CatG = cathepsin G; DNA = deoxyribonucleic acid; FEV1 = forced expiratory volume in one second; IL = interleukin; MPO = myeloperoxidase; NE = neutrophil elastase; PR3 = proteinase 3; TNF-α = tumor necrosis factor α.