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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Changes in sputum viscoelastic properties, inflammation markers and the proteome in primary ciliary dyskinesia are less severe than in cystic fibrosis at baseline, but comparable to cystic fibrosis after starting elexacaftor/tezacaftor/ivacaftor therapy https://bit.ly/41zp2Mb

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Abstract

Background Primary ciliary dyskinesia (PCD) and cystic fibrosis (CF) are muco-obstructive lung diseases that are caused by distinct genetically determined defects in mucociliary clearance; however, knowledge on the relative severity of airway mucus dysfunction and chronic inflammation remains limited. The aim of this study was therefore to compare sputum viscoelastic properties, inflammation markers and the proteome between patients with PCD and patients with CF before and under elexacaftor/tezacaftor/ivacaftor (ETI) therapy.

Methods We compared sputum rheology, inflammation markers and the proteome in 42 clinically stable adolescent and adult patients with PCD, 40 patients with CF with at least one *F508del* allele before (baseline) and 3 months after initiation of ETI, and 15 age-matched healthy controls.

Results The elastic modulus (G') and viscous modulus (G'') of PCD sputum was increased compared to healthy controls ($p<0.001$), lower than in CF at baseline ($p<0.001$) and similar to CF on ETI. Inflammation markers in PCD sputum including neutrophil elastase, free DNA, myeloperoxidase, interleukin (IL)-1β and IL-8 were also increased compared to healthy controls (all $p<0.001$), lower than in CF at baseline ($p<0.05$ to $p<0.001$) and comparable to CF on ETI. Similarly, changes in the sputum proteome were less pronounced in PCD compared to CF at baseline, but comparable between PCD and CF on ETI.

Conclusions Clinically stable patients with PCD show changes in sputum viscoelastic properties, inflammation markers and the proteome that are less severe than in patients with CF at baseline, but comparable to CF patients on ETI therapy.

Introduction

Primary ciliary dyskinesia (PCD) and cystic fibrosis (CF) are rare genetic lung diseases caused by distinct molecular and cellular defects [1, 2]. PCD is caused by mutations in more than 50 genes that are essential for normal structure and function of respiratory cilia, thus leading to primary defects in ciliary function [2–5]. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene

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encoding an epithelial chloride channel essential for chloride/fluid secretion and airway surface hydration, thus leading to increased concentration and viscoelasticity of airway mucus [6]. Primary defects in ciliary function in PCD, as well as primary abnormalities in mucus function in CF, cause impaired mucociliary clearance that triggers a pathogenetic cascade that evolves into a "vicious vortex" of airway mucus plugging, chronic polymicrobial infection, neutrophilic inflammation and structural airway damage [5, 7–9]. The secretion of the neutrophil serine proteases (NSPs) neutrophil elastase (NE), cathepsin G (CatG) and proteinase 3 (PR3) by activated neutrophils causes proteolytic damage that plays a key role in the onset and progression of bronchiectasis characteristic of lung disease in PCD and CF [9–14]. Recent studies of patients with CF treated with the triple combination CFTR modulator therapy elexacaftor/tezacaftor/ivacaftor (ETI) showed that pharmacological restoration of CFTR chloride channel function reduces neutrophilic airway inflammation including NSP activity, although without reaching levels close to healthy [15, 16]. Of note, markers of neutrophilic inflammation including NE and interleukin (IL)-1β were reported to induce goblet cell metaplasia and mucus hypersecretion, which may also cause increased viscoelasticity of airway mucus that may aggravate mucociliary dysfunction in PCD [6, 17, 18]. While previous studies demonstrated neutrophilic airway inflammation with elevated NE activity in CF and PCD, the viscoelastic properties of sputum from patients with PCD have not been studied and a comparison of the relative levels of NSP and other inflammation markers in PCD *versus* CF patients without and after initiation of CFTR modulator therapy has not been performed.

The aim of this study was therefore to compare sputum viscoelastic properties and airway inflammation markers in clinically stable adolescent and adult patients with PCD and age-matched patients with CF at baseline and under triple combination therapy with ETI. To achieve this goal, we investigated sputum viscoelastic properties by rheology, determined levels of key inflammation markers including NSP activity, DNA, myeloperoxidase (MPO), interleukin (IL)-1β and IL-8, and conducted proteomic analyses in 42 patients with PCD, 40 patients with CF at baseline and after initiation of ETI therapy, and 15 age-matched healthy controls.

Methods

Additional details on methods are provided in the supplementary material.

Study design and participants

This prospective observational study was approved by the ethics committee of Charité – Universitätsmedizin Berlin (EA2/220/18, EA2/143/19). Written informed consent was obtained from all participants, their parents or legal guardians. Patients with PCD had to be clinically stable at the time of sputum collection. Age-matched patients with CF were at least 12 years old, eligible for CFTR modulator therapy with ETI (homozygous for *F508del* or compound heterozygous for *F508del* and a minimal function mutation) and provided paired sputum samples before and after initiation of ETI therapy. CF patients were a subgroup of the previously described MODULATE-CF cohort (ClinicalTrials.gov: NCT04732910) [16], and were matched with PCD patients for age, gender and the existence of matched sputum samples at baseline and at 3 months after initiation of ETI therapy for paired analysis. A complete list of inclusion and exclusion criteria is detailed in the supplementary material. Healthy controls were age- and sex-matched non-smoking volunteers without any medical history of respiratory disease. Sputum samples from CF patients were analysed at baseline and 3 months after initiation of therapy with the approved dose of elexacaftor 200 mg and tezacaftor 100 mg every 24 h in combination with ivacaftor 150 mg every 12 h. Due to limited amount of sputum in some patients not all measurements could be performed with every sputum sample.

Sputum collection

Sputum samples from patients with PCD and patients with CF were obtained after spontaneous expectoration. In healthy subjects, sputum expectoration was induced by hypertonic saline inhalation (sodium chloride 3–6%). Rheology measurements were performed on fresh sputum samples that were immediately placed on ice. For all other analyses the remaining samples were stored at −80 °C.

Rheology

Rheological measurements were conducted using a rheometer with a cone–plate geometry (Kinexus Pro+; Netzsch, Selb, Germany) as previously described [19]. The elastic modulus (storage modulus, G') and viscous modulus (loss modulus, G'') were derived from the linear viscoelastic region of the amplitude sweep.

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Inflammation markers

IL-1β, IL-6, IL-8 and tumour necrosis factor (TNF)-α concentrations in cell-free sputum supernatants were determined by cytometric bead array kits (BD Biosciences, San Diego, CA, USA), MPO concentration was measured by ELISA (Enzo Life Sciences, Farmingdale, NY, USA) and DNA concentration was measured using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Free NE, CatG and PR3 activity was measured as previously described [10, 20–22].

Proteomics

Sputum samples were solubilised in 2% SDS buffer at 95°C for 10 min. Proteins were reduced, alkylated and treated with Benzonase. Samples were cleaned-up using a single-pot solid-phase enhanced sample preparation protocol, followed by PNGase F and trypsin treatment overnight. Peptide samples were desalted and 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 mode as previously described [23].

Magnetic resonance imaging

Magnetic resonance imaging (MRI) of the lungs was performed in a subgroup of patients with PCD and patients with CF, and images were analysed for abnormalities in lung morphology and perfusion including the presence and extent of bronchiectasis using an established morpho-functional MRI score [24, 25] as detailed in the supplementary material.

Statistical analysis

Data were analysed using Prism version 9.5.1 (GraphPad, Boston, MA, USA) or R version 4.2.2 (www.r-project.org). Clinical data are presented as mean with standard deviation and tested by the t-test. Data from measurements in sputum samples from healthy subjects, PCD patients and CF patients are presented as median with interquartile range and were compared by the Mann–Whitney test. Changes in CF sputum between baseline and 3 months after initiation of ETI were tested with the Wilcoxon matched-pairs signed-rank test. For proteomics analysis the limma moderated t-test or F-test was used. The Benjamini–Hochberg method was used for multiple comparisons correction. $p<0.05$ was accepted to indicate statistical significance.

Results

Characteristics of study population

In total, 42 clinically stable adolescent and adult patients with PCD, and 40 age-matched patients with CF and 15 healthy controls were included in this study, and their demographics and clinical characteristics are summarised in table 1. Patients with PCD had a mean±SD age of 27.2±14.7 years at sputum collection. Outer dynein arm defects with homozygous or compound heterozygous mutations in the dynein axonemal heavy chain 5 (*DNAH5*) (19.1%) and the dynein axonemal intermediate chain 1 (*DNAI1*) (12.0%) genes were the most frequent PCD-causing genotypes [2]. PCD genotypes are provided in supplementary table S1. CF patients from the MODULATE-CF cohort had a mean±SD age of 29.9±9.5 years at baseline sputum collection; 50.0% were *F508del* homozygous and 50% compound heterozygous for *F508del* and a minimal function mutation (table 1). *CFTR* genotypes are indicated in supplementary table S2. As expected from previous studies, forced expiratory volume in 1 s (FEV_1) % predicted, body mass index and sweat chloride concentration were improved in CF patients by ETI therapy (table 1) [16, 26]. The prevalence of bronchiectasis detected by MRI was 97.1% in PCD patients and 100% in CF patients. The extent of bronchiectasis and airway mucus plugging, as determined by the MRI bronchiectasis/wall thickening subscore and mucus plugging subscore, respectively, was lower in PCD compared to CF at baseline, but comparable to CF on ETI (supplementary table S4).

Comparison of sputum viscoelastic properties in PCD and CF at baseline and on ETI therapy

To determine disease-specific differences in sputum viscoelasticity we performed rheological measurements and determined the elastic modulus (G'), viscous modulus (G'') and the resulting effective rheological mesh size (ξ) in sputum samples of 39 patients with PCD, 40 patients with CF at baseline and 3 months after initiation of ETI, and 10 healthy controls. In all three groups, G' predominated over G'', indicating a gel-like behaviour of sputum (figure 1a and b). G' and G'' of induced sputum from healthy controls were similar to G' and G'' of mucus collected from endotracheal tubes of healthy individuals without hypertonic saline induction [27]. In PCD, G' and G'' were increased and the mesh size was decreased compared to healthy controls (figure 1). In CF sputum prior to ETI, G' and G'' were increased and the mesh size was decreased compared to sputum from PCD patients. On ETI therapy, G' and G'' of CF sputum were reduced and the mesh size increased to levels comparable to values observed for PCD

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TABLE 1 Clinical characteristics of healthy controls, patients with primary ciliary dyskinesia (PCD), and patients with cystic fibrosis (CF) at baseline and after initiation of elexacaftor/tezacaftor/ivacaftor (ETI)

	Healthy controls	PCD patients	CF patients at baseline	CF patients with 3 months ETI
Individuals	15	42	40	40
Age (years)	32.5±5.7	27.2±14.9	29.9±9.5	30.2±9.5
Female	13 (87.0)	21 (51.2)	25 (62.5)	25 (62.5)
Organ laterality defect		18 (42.9)		
PCD mutation subgroups (ultrastructural defect category)				
ODA		17 (40.5)		
IDA		1 (2.4)		
ODA+IDA		6 (14.3)		
CC/RS		5 (12.0)		
MTD		6 (14.3)		
Normal ciliary ultrastructure		4 (9.5)		
Unknown genotype[#]		1 (2.4)		
CF genotype				
F508del/F508del			20 (50.0)	20 (50.0)
F508del/MF			20 (50.0)	20 (50.0)
No prior CFTR modulator			24 (60.0)	
TEZ/IVA therapy			12 (30.0)	
LUM/IVA therapy			4 (10.0)	
Sweat chloride (mmol·L^{-1})			95.8±13.3	44.2±20.4
Change±SD				−51.6±17.3
p-value				<0.001
FEV_1 % pred		78.9±23.4	41.7±20.4	51.2±23.8
Change±SD				9.5±8.3
p-value				<0.001
BMI (kg·m^{-2})	24.2±3.2	23.0±5.1	19.3±2.4	20.5±2.2
Change±SD				1.2±0.9
p-value				<0.001
Respiratory culture				
Haemophilus influenzae		11 (26.8)	0 (0.0)	1 (2.5)
Staphylococcus aureus		13 (31.0)	21 (52.5)	19 (47.5)
Streptococcus pneumoniae		2 (4.8)	0 (0.0)	0 (0.0)
Pseudomonas aeruginosa		19 (45.2)	30 (75.0)	30 (75.0)
Mycobacteria (MOTT)		1 (2.4)	0 (0.0)	0 (0.0)

Data are presented as n, mean±SD or n (%), unless otherwise stated. ODA: outer dynein arm; IDA: inner dynein arm; CC/RS: central complex/radial spokes defect; MTD: microtubular disorganisation; MF: minimal function; CFTR: cystic fibrosis transmembrane conductance regulator; TEZ: tezacaftor; IVA: ivacaftor; LUM: lumacaftor; FEV_1: forced expiratory volume in 1 s; BMI: body mass index; MOTT: mycobacteria other than tuberculosis. [#]: PCD diagnosis according to American Thoracic Society criteria [53]. p-values describe the changes between paired measurements from baseline and ETI therapy.

patients; however, rheological parameters remained significantly changed compared to healthy controls (figure 1). To determine the potential impact of inhaled hypertonic saline on sputum rheology, we compared viscoelastic properties of spontaneously expectorated *versus* induced sputum from patients with PCD and patients with CF, and did not observe differences in G' or G'' (supplementary figure S1). Further, hypertonic saline as standard care for PCD and CF, as well as recombinant human DNase treatment in CF, had no effect on sputum viscoelastic properties (supplementary figure S2).

Comparison of airway inflammation markers in PCD and CF at baseline and on ETI therapy

To study disease-specific differences in airway inflammation, we measured the concentration of free DNA, MPO, IL-1β, IL-6, IL-8 and TNF-α, *i.e.* key inflammation markers implicated in lung disease progression [18, 28–30], in sputum supernatants from 40 patients with PCD, 40 patients with CF at baseline and after 3 months on ETI, and 12 healthy controls (figure 2). In PCD, DNA, MPO, IL-1β, IL-8 and TNF-α levels were elevated compared to healthy controls, but lower compared to CF at baseline (figure 2a–d and f). In CF patients on ETI therapy, DNA, MPO, IL-1β and IL-8 were reduced to levels similar to those observed in PCD, but remained elevated compared to healthy controls (figure 2a–d). TNF-α showed a trend towards

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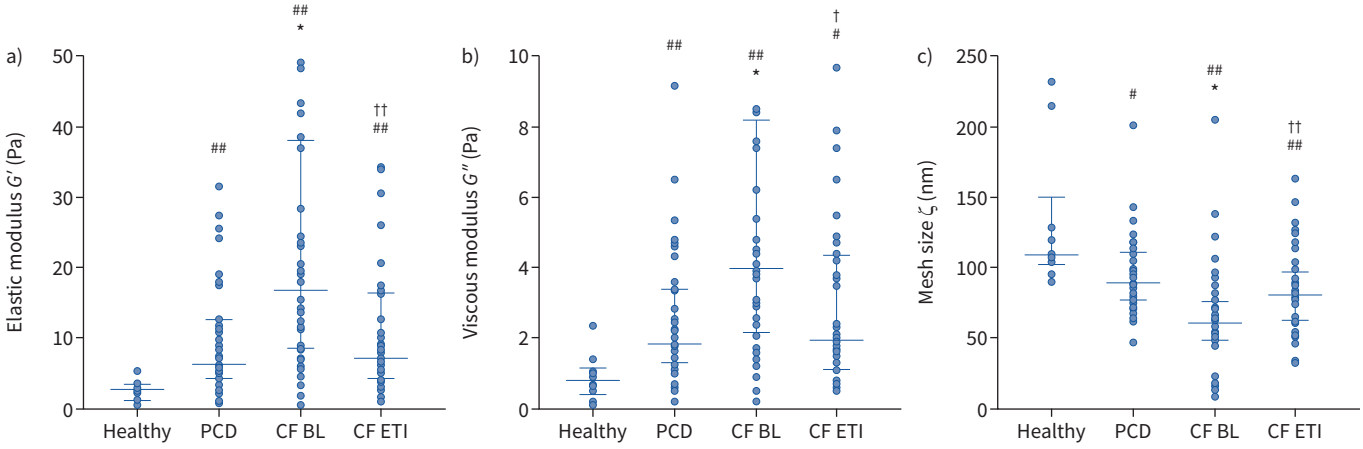

FIGURE 1 Changes in viscoelastic properties of sputum of patients with primary ciliary dyskinesia (PCD) are comparable to changes in patients with cystic fibrosis (CF) on elexacaftor/tezacaftor/ivacaftor (ETI) therapy. Viscoelastic properties of sputum samples were determined by a cone and plate rheometer. Measurements of a) elastic modulus G', b) viscous modulus G'' and c) mesh size ξ of sputum from healthy controls (n=10), patients with PCD (n=39), and patients with CF at baseline (BL) and at 3 months after initiation of ETI therapy (n=40). Bars represent the group median; error bars represent the 25th and 75th percentiles (interquartile range). *: p<0.001 compared with PCD; †: p<0.05, ††: p<0.01 compared with BL; #: p<0.01, ##: p<0.001 compared with healthy controls.

a decrease on ETI therapy, but was still increased compared to PCD patients and healthy controls (figure 2f). Sputum IL-6 was not changed in PCD compared to healthy controls (figure 2e). CF patients showed elevated sputum levels of IL-6 at baseline, which increased after 3 months of ETI therapy.

Comparison of airway protease burden in PCD and CF at baseline and on ETI therapy

An imbalance between proteases and anti-proteases, particularly an elevated NSP activity, constitutes a key risk factor for the initiation and progression of lung disease in both PCD and CF [9–11, 31]. To assess disease-specific differences in protease burden, we measured free activity of NE, CatG and PR3 in sputum supernatants from 42 PCD patients, 40 CF patients at baseline and after 3 months on ETI, and 12 healthy controls (figure 3). Free NE, CatG and PR3 activity was increased in PCD compared to healthy controls and showed a further increase in CF at baseline (figure 3a–c). On ETI therapy, free NE, CatG and PR3 activity in CF sputum was similar to that observed in PCD, but remained increased compared to healthy controls (figure 3a–c).

Relationship between changes in viscoelastic properties and airway inflammation markers

To determine the relationship between the changes in sputum viscoelastic properties and airway inflammation, we correlated G' and G'' with individual inflammation markers (supplementary figures S3 and S4). These analyses show a direct correlation of G' and G'' with DNA, IL-1β, IL-8, TNF-α and free NSP activities, indicating that abnormal mucus is associated with more severe airway inflammation. On the other hand, lung disease severity determined by FEV_1 % predicted and the presence or absence of specific pathogens, such as *Haemophilus influenzae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, had a relatively small impact on changes in sputum viscoelasticity and inflammation markers in PCD and CF, suggesting that the observed variability in sputum outcome measures may also be linked to sputum as a biospecimen that may originate from a more or less severely affected region of the airways (supplementary tables S5 and S6).

Comparison of sputum proteome in PCD and CF at baseline and on ETI therapy

For an unbiased comparison of the sputum proteome, we conducted label-free shotgun proteomics of sputum samples from 29 patients with PCD, 35 patients with CF before and on ETI therapy, and 15 healthy controls. In total, 1149 proteins were detected, with 690 (60.1%) proteins being significantly different between all four groups (figure 4a). The complete list of all identified proteins is included in the supplementary material. Hierarchical cluster analysis identified two major clusters (figure 4a). Cluster 1 contains proteins that were detectable at higher levels in PCD and CF sputum compared to healthy individuals and were associated with inflammatory processes, *e.g.* leukocyte/myeloid-mediated immunity and phagocytosis (figure 4a). Cluster 2 includes proteins with overall decreased abundance in sputum from patients with PCD and patients with CF compared to healthy controls, and is enriched in proteins involved

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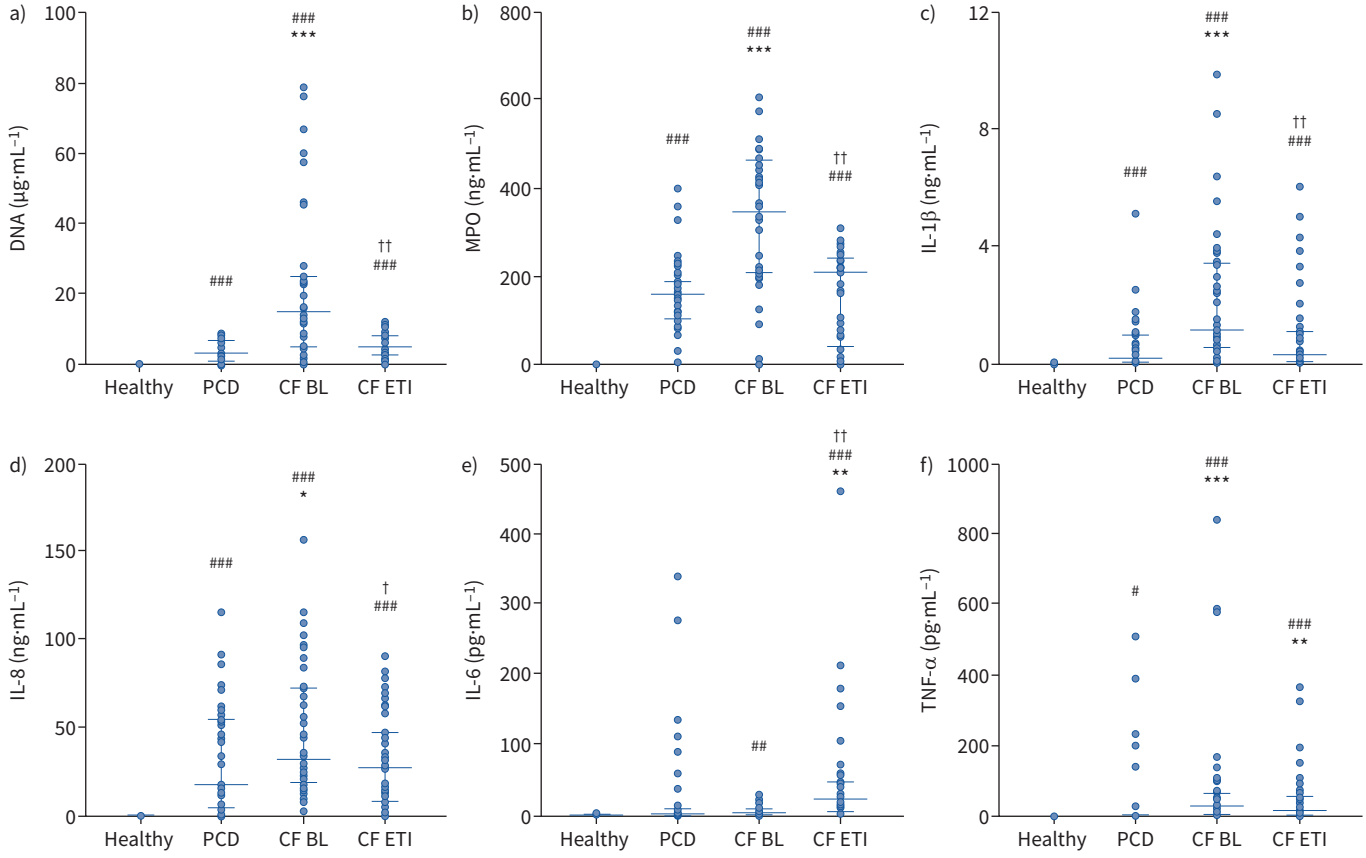

FIGURE 2 Inflammation markers in sputum of patients with primary ciliary dyskinesia (PCD) are comparable to patients with cystic fibrosis (CF) on elexacaftor/tezacaftor/ivacaftor (ETI) therapy. Measurements of a) DNA, b) myeloperoxidase (MPO), c) interleukin (IL)-1β, d) IL-8, e) IL-6 and f) tumour necrosis factor (TNF)-α in sputum supernatant from healthy controls (n=10–12), patients with PCD (n=36–41), and patients with CF at baseline (BL) and at 3 months after initiation of ETI therapy (n=36–40). Bars represent the group median; error bars represent the 25th and 75th percentiles (interquartile range). *: p<0.05, **: p<0.01, ***: p<0.001 compared with PCD; †: p<0.05, ††: p<0.001 compared with BL; #: p<0.05, ##: p<0.01, ###: p<0.001 compared with healthy controls.

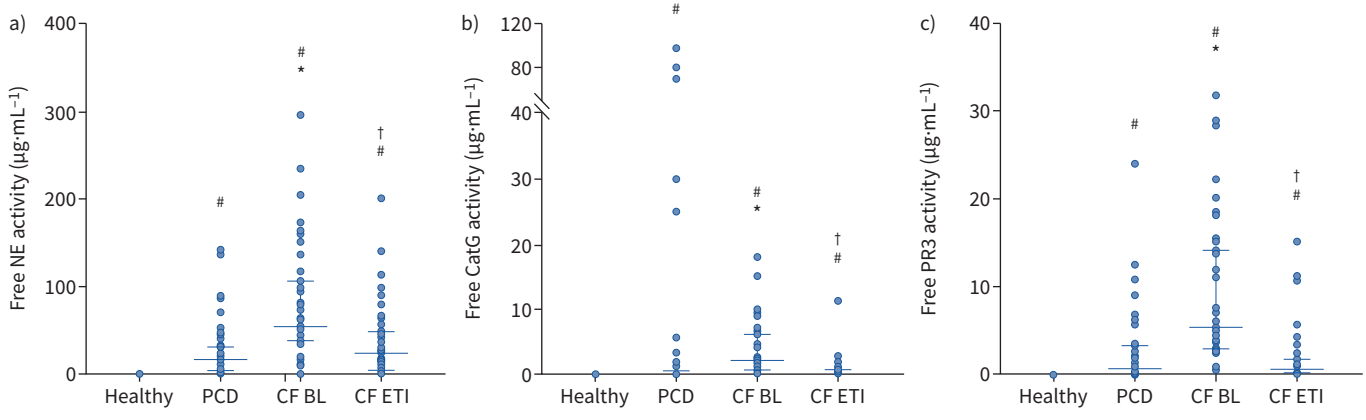

FIGURE 3 Neutrophil serine protease activity in sputum of patients with primary ciliary dyskinesia (PCD) is comparable to patients with cystic fibrosis (CF) on elexacaftor/tezacaftor/ivacaftor (ETI) therapy. Measurements of free activity of a) neutrophil elastase (NE), b) cathepsin G (CatG) and c) proteinase 3 (PR3) in cell-free sputum supernatants from healthy controls (n=12–13), patients with PCD (n=40–42), and patients with CF at baseline (BL) and at 3 months after initiation of ETI therapy (n=40). Bars represent the group median; error bars represent the 25th and 75th percentiles (interquartile range). *: p<0.001 compared with PCD; †: p<0.001 compared with BL; #: p<0.001 compared with healthy.

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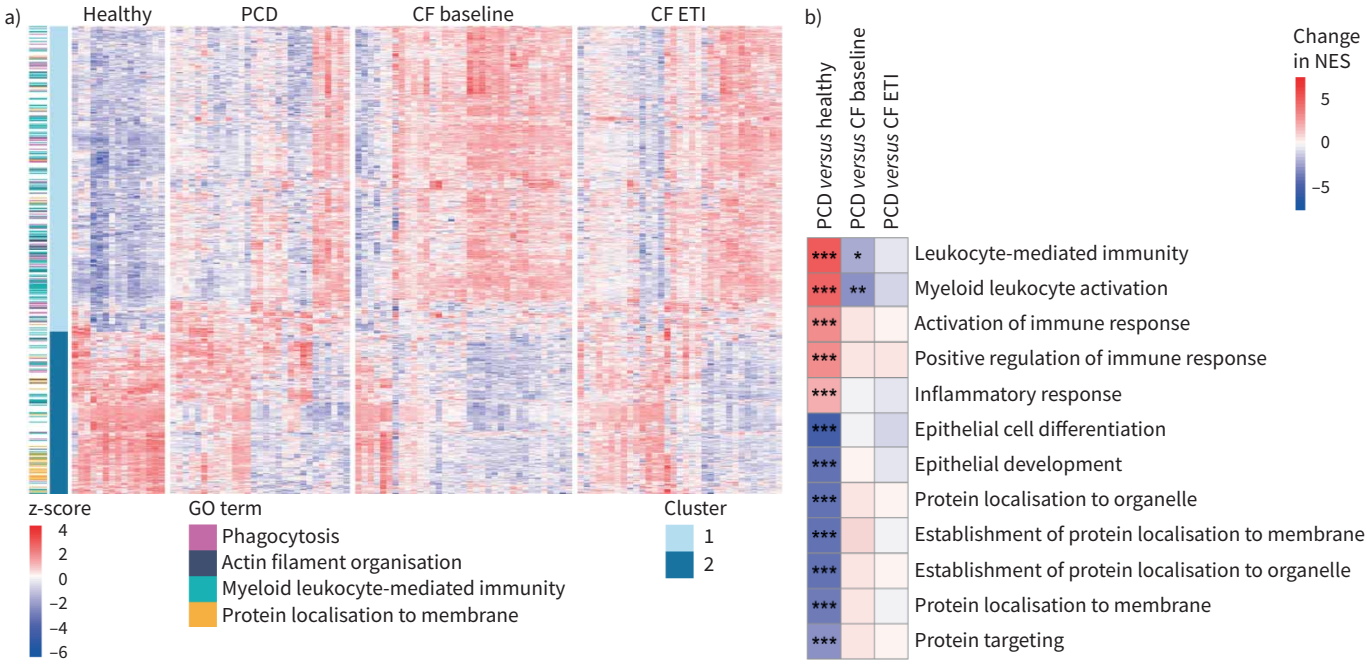

FIGURE 4 Changes in the sputum proteome of patients with primary ciliary dyskinesia (PCD) are comparable to changes in patients with cystic fibrosis (CF) on elexacaftor/tezacaftor/ivacaftor (ETI) therapy. a) Heatmap of significantly differentially expressed proteins (cut-off p<0.01) in sputum from healthy controls (n=15), patients with PCD (n=29), and patients with CF at baseline and at 3 months after initiation of ETI therapy (n=35). Hierarchical clustering identified two major clusters with upregulated proteins (cluster 1) and downregulated proteins (cluster 2) in PCD and CF compared to healthy controls. Proteins contributing to the most representative enriched Gene Ontology (GO) terms are highlighted in the first column. b) Single-sample gene set enrichment analysis of GO terms in PCD sputum compared to healthy controls (first column), CF baseline (second column) and CF after 3 months ETI treatment (third column) ordered by change in normalised enrichment score (NES) (false discovery rate <0.01). *: p<0.05; **: p<0.01; ***: p<0.001.

in biological processes such as protein localisation to membrane/organelle and epithelial cell differentiation (figure 4a).

Comparative analysis of protein expression revealed that 598 (52.0%) proteins were significantly different between healthy controls and patients with PCD, of which 388 showed a higher and 210 a lower abundance in PCD compared to healthy controls (supplementary figure S5a). Single-sample gene set enrichment analysis (ssGSEA) of Gene Ontology (GO) terms showed a high enrichment of the GO terms leukocyte-mediated immunity, myeloid leukocyte activation and activation/positive regulation of immune response in PCD, whereas GO terms linked to epithelium development, protein targeting and localisation to membrane were enriched in healthy controls (figure 4b). The comparison between the PCD and CF sputum proteome at baseline revealed 447 (38.9%) proteins to be significantly different (supplementary figure S5b). Enrichment of both inflammation-associated GO terms, leukocyte-mediated immunity and myeloid leukocyte activation, was significantly higher in CF than in PCD sputum (figure 4b and supplementary figure S6d). The complete list of significantly different GO terms is included in supplementary figure S7. Protein expression profiles in PCD sputa were similar to CF on ETI therapy with only 63 (5.5%) significantly different proteins between PCD and CF (supplementary figure S5c). Correspondingly, $\log_2$ fold change analysis of protein abundances detected proteins with a greater fold change in the comparisons of PCD *versus* healthy controls and PCD *versus* CF at baseline (supplementary figure S5a and c) than in the comparison of PCD with CF on ETI (supplementary figure S5b). Taken together, these data show that the sputum proteome of PCD patients is rather similar to that of patients with CF on ETI therapy.

Discussion

PCD and CF are caused by distinct genetic defects in the mucociliary clearance system. However, our understanding of the relative severity of mucus dysfunction and chronic airway inflammation in these two muco-obstructive lung diseases remains limited. This is the first study to compare changes in sputum

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viscoelastic properties, inflammation markers and the sputum proteome in clinically stable adolescent and adult patients with PCD with age-matched patients with CF before and after initiation of ETI therapy. This study shows that compared to healthy controls, sputum from patients with PCD is characterised by increased viscoelasticity, increased markers of neutrophilic inflammation and protease burden, and an altered sputum proteome. Overall, the changes in mucus properties, inflammation markers and the proteome profile found in PCD sputum were less severe than in patients with CF at baseline, but comparable to residual abnormalities observed in CF after initiation of ETI therapy. Collectively, these data provide novel information on relative abnormalities in mucus properties and severity of neutrophilic inflammation and protease burden in PCD *versus* CF, including patients with CF in whom CFTR chloride channel function is pharmacologically restored by highly effective CFTR modulators.

In CF, increased viscoelasticity of airway mucus is primarily caused by deficient CFTR-mediated chloride and fluid secretion leading to mucus hyperconcentration, which is well established as a key mechanism of mucociliary dysfunction in CF [32–35]. In addition to mucus hyperconcentration, increased cross-linking of mucins *via* disulfide bonds caused by oxidation of free thiols under conditions of neutrophilic inflammation was identified as an independent mechanism leading to pathological mucus in CF [36, 37]. Conversely, mucociliary dysfunction in PCD has been primarily related to ciliary dysfunction [2]. Our data show for the first time that viscoelastic properties of airway mucus are also fundamentally altered in PCD with a characteristic increase in the elastic modulus (G') and viscous modulus (G'') and a decrease in mesh size of the mucus gel. Similar to prior reports in CF, we found that G' is consistently higher than G'' in PCD. Since this G' dominance is a hallmark of a cross-linked gel, our data indicate that increased mucin cross-linking also contributes to mucus dysfunction in PCD. Further, several factors released in neutrophilic inflammation, including NE and IL-1β, were shown to induce mucin hypersecretion [9, 17, 18, 38], and thus may increase mucus concentration and contribute to increased viscoelasticity of airway mucus in PCD. Our data indicate that in addition to ciliary dysfunction, mucus dysfunction may also contribute to the pathogenesis of impaired mucociliary clearance in PCD, where increased viscoelasticity may impede back-up mechanisms of mucus clearance such as cough clearance and cilia-independent gas liquid transport [39, 40]. The relevance of mucus dysfunction in PCD is also supported by the high prevalence of airway mucus plugging detected by MRI. As stagnant mucus causes airflow limitation and hypoxia, and provides a constant nidus for chronic airway infection and inflammation, our results also provide a rationale for therapeutic targeting of mucus dysfunction as a treatable trait in PCD [39, 41]. Current strategies in this direction include treatment with inhaled idrevloride, a long-acting inhibitor of the epithelial sodium channel ENaC, in combination with hypertonic saline to improve mucus hydration [42], and the development of novel mucolytics that reduce mucus cross-linking [33, 36]. Finally, our data show that the residual abnormalities in viscoelastic properties as well as residual mucus plugging detected by MRI in adolescent and adult CF patients treated with ETI are comparable to those observed in PCD. These data suggest that these novel mucoactive and mucolytic strategies remain relevant for CF, not only in the ∼10% of patients who are genetically ineligible or do not tolerate CFTR modulators, but also in the much larger group of CF patients who can benefit from CFTR modulators but have irreversible lung damage with bronchiectasis.

Neutrophilic inflammation with increased protease burden due to release of NE and other NSPs has been identified as a key risk factor for the development of bronchiectasis and lung function decline in the broader non-CF bronchiectasis population as well as in patients with CF [9, 10, 31, 43, 44]. Our comparative studies of sputum inflammation markers and proteome profiles indicate that the severity of neutrophilic inflammation in clinically stable PCD patients is lower than in CF patients at baseline, but comparable to CF patients on CFTR modulator therapy with ETI. This pattern is observed for key pro-inflammatory cytokines that orchestrate neutrophilic airway inflammation such as IL-1β, IL-8 and TNF-α [18, 28], as well as all three NSPs, *i.e.* NE, CatG and PR3, that play a key role in the pathogenesis of protease–antiprotease imbalance leading to proteolytic airway damage, progressive bronchiectasis and lung function decline [9–11, 31, 43–45]. Of note, IL-6 levels in sputum were not different in PCD *versus* CF at baseline, but increased in CF patients on ETI therapy. Based on recent work in the gastrointestinal tract, where IL-6 has been linked to epithelial homeostasis [46, 47], we therefore speculate that IL-6 may represent a biomarker of airway epithelial regeneration on ETI therapy. A similar pattern was also observed in our proteomics analyses as an unbiased approach to study airways disease at the molecular level. As shown by comparative analysis of protein abundances, as well as ssGSEA of GO terms, changes in PCD sputum, including proteins related to leukocyte-mediated immunity and myeloid leukocyte activation, were less pronounced than in CF sputum at baseline, but comparable to CF after initiation of ETI therapy. These findings are in line with a previous report that compared these inflammation markers and activities of NE, CatG and PR3 in CF patients before and after initiation of ETI with patients with non-CF bronchiectasis [15]. Notably, the proteomic signature of a subset of CF patients at baseline was more similar to healthy

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individuals. Those CF patients tended to have a lower sputum elastic modulus (G') and viscous modulus (G''), and exhibited lower levels of airway inflammation markers, supporting the sensitivity of proteomics to detect changes and commonalities between different disease and treatment conditions (supplementary table S8). Collectively, these studies suggest that with pharmacological restoration of CFTR function, the airway pathobiology in CF may resemble more closely that in non-CF bronchiectasis, where the disease process is perpetuated by irreversible airway damage rather than CFTR dysfunction. This notion supports that novel anti-inflammatory therapies such as oral inhibitors of dipeptidyl peptidase 1 (also known as cathepsin C) that inhibit the activity of all three NSPs and were recently shown to reduce pulmonary exacerbations in patients with non-CF bronchiectasis including PCD [31, 45, 48–50] should also be trialled in patients with CF, including those that are treated with CFTR modulators.

This study has limitations. Our studies of ETI effects were limited to a 3-month time-point and studies with a longer follow-up will be needed to determine when optimal benefits are reached and when disease progression may be observed in CF patients due to persistent bronchiectasis and residual mucus dysfunction, inflammation and infection on CFTR modulator therapy. We only included adolescent and adult patients with PCD or CF with chronic lung disease that were able to expectorate sputum and it remains unknown whether the abnormalities and disease-specific differences detected in sputum viscoelasticity, inflammation markers and the proteome in PCD and CF are also present in patients with milder lung disease, including children. Therefore, future studies in younger patients with less severe lung disease, ideally prior to the onset of irreversible structural airway damage, are needed to clarify the extent to which airway mucus dysfunction and inflammation in PCD and CF are early consequences of the underlying genetic defects or secondary changes related to an independent pathogenetic mechanisms (*i.e.* the “vicious vortex”) driven by bronchiectasis. Such studies will have to rely on induced sputum that can be obtained from early childhood [51]. In this context, our data on viscoelastic properties of induced sputum in healthy individuals were comparable to mucus collected from endotracheal tubes from healthy individuals without prior induction with hypertonic saline [27]. Further, viscoelastic properties and inflammation markers were comparable in spontaneously expectorated *versus* induced sputum from PCD patients and CF patients, which may be explained by rapid absorption of the added fluid by the airway epithelium [52] and supports the suitability of induced sputum for such studies. Further, it will be important to determine how mucus properties and inflammation markers change over time, including episodes of pulmonary exacerbations, and how these changes are related to patient-reported outcome measures of health-related quality of life.

In summary, our data support that changes in mucus properties and neutrophilic airway inflammation in PCD are less severe than in CF patients not treated with CFTR modulators, but comparable to CF patients on ETI therapy. These results provide novel information on the relative severity of key contributors to the vicious vortex underlying the complex *in vivo* pathogenesis of chronic airways disease in PCD and CF, and support therapeutic targeting of mucus plugging and neutrophilic inflammation in both diseases, including CF patients who can benefit from CFTR modulators.

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