SPLUNC1: a novel marker of cystic fibrosis exacerbations

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Shareable abstract (@ERSpublications)
Sputum concentrations of the secreted airway protein SPLUNC1 decrease during CF exacerbations. Lower SPLUNC1 levels in stable participants portend a significantly increased risk of exacerbation which could inform therapeutic interventions. https://bit.ly/3uDGDzF

Cite this article as: Khanal S, Webster M, Niu N, *et al*. SPLUNC1: a novel marker of cystic fibrosis exacerbations. *Eur Respir J* 2021; 58: 2000507 [DOI: 10.1183/13993003.00507-2020].

This article has supplementary material available from erj.ersjournals.com

This article has an editorial commentary: https://doi.org/10.1183/13993003.01569-2021

Received: 2 March 2020
Accepted: 29 March 2021

Abstract

Background Acute pulmonary exacerbations (AE) are episodes of clinical worsening in cystic fibrosis (CF), often precipitated by infection. Timely detection is critical to minimise morbidity and lung function declines associated with acute inflammation during AE. Based on our previous observations that airway protein short palate lung nasal epithelium clone 1 (SPLUNC1) is regulated by inflammatory signals, we investigated the use of SPLUNC1 fluctuations to diagnose and predict AE in CF.

Methods We enrolled CF participants from two independent cohorts to measure AE markers of inflammation in sputum and recorded clinical outcomes for a 1-year follow-up period.

Results SPLUNC1 levels were high in healthy controls (n=9, 10.7 $\mu g \cdot mL^{-1}$), and significantly decreased in CF participants without AE (n=30, 5.7 $\mu g \cdot mL^{-1}$; p=0.016). SPLUNC1 levels were 71.9% lower during AE (n=14, 1.6 $\mu g \cdot mL^{-1}$; p=0.0034) regardless of age, sex, CF-causing mutation or microbiology findings. Cytokines interleukin-1β and tumour necrosis factor-α were also increased in AE, whereas lung function did not decrease consistently. Stable CF participants with lower SPLUNC1 levels were much more likely to have an AE at 60 days (hazard ratio (HR)±SE 11.49±0.83; p=0.0033). Low-SPLUNC1 stable participants remained at higher AE risk even 1 year after sputum collection (HR±SE 3.21±0.47; p=0.0125). SPLUNC1 was downregulated by inflammatory cytokines and proteases increased in sputum during AE.

Conclusion In acute CF care, low SPLUNC1 levels could support a decision to increase airway clearance or to initiate pharmacological interventions. In asymptomatic, stable patients, low SPLUNC1 levels could inform changes in clinical management to improve long-term disease control and clinical outcomes in CF.

Background

Cystic fibrosis (CF) is a multisystem, autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1–4]. CF acute pulmonary exacerbations (AE) are generally reversible episodes of acute deterioration, associated with increased morbidity and worsening quality of life [5–9]. AE are frequently triggered by respiratory viruses, but also by oropharyngeal flora and bacterial respiratory pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [10–12]. Increased lung inflammation during AE, manifested as higher immune cell counts and rising concentrations of airway cytokines and proteases, contribute to tissue injury and disease progression [7, 13]. Increased AE frequency decreases CF survivorship and accelerates lung function decline [14, 15]. Importantly, delays in AE detection and treatment may have long-term effects on lung function recovery and response to antibiotic treatment [16, 17]. These observations suggest that early AE detection could help improve clinical outcomes in CF.

Early or mild AE presentations can go undetected during routine visits [7, 18, 19], exacerbating an already variable AE diagnostic approach among healthcare providers [20] and impacting treatment outcomes [21]. Biomarkers of airway inflammation or lung function, such as forced expiratory volume in 1 s (FEV_1), are routinely used to support AE diagnosis and management [18, 19]. However, FEV_1 changes often occur as a late consequence of AE, limiting its clinical use in early detection [22]. Inflammatory cytokines (*e.g.* interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF)-α) have also been linked to AE, but there are limited data on their ability to predict these events [23–25].

Our group and others previously demonstrated that airway concentrations of host defence protein short palate lung nasal epithelium clone 1 (SPLUNC1) are closely regulated by inflammatory signals and proteases [26–29]. SPLUNC1 is primarily expressed by nonciliated epithelial and mucus cells of the upper and proximal lower respiratory tract [30–32]. SPLUNC1 is present at low levels in extrapulmonary tissues and myeloid cells [30, 32]. SPLUNC1 has antimicrobial, immunomodulatory and ion transport properties that are highly relevant to CF health, which may be disrupted at baseline and during AE [28, 33, 34].

SPLUNC1 decreases within hours of exposure to inflammation, irritants or pathogens [33], and it is differentially regulated in lung disease [27, 28, 33, 35–39]. In CF, previous studies have shown low levels in respiratory secretions [28], but increased bronchial SPLUNC1 staining in advanced disease [40]. Recently, genome-wide association studies showed that *SPLUNC1* expression was higher in stable CF patients compared to healthy controls, but lower in CF patients with more severe disease [41, 42]. In asthma and active smokers, lower SPLUNC1 levels correlate with increased inflammation [35, 37, 38]; however, studies of its regulation in COPD have been inconclusive [27, 39]. Beyond airway disease, SPLUNC1 dysregulation has been reported in idiopathic pulmonary fibrosis and respiratory malignancies [33, 36, 43]. The variable relationship between SPLUNC1 regulation, inflammation and underlying lung disease suggests that SPLUNC1 has a role as rheostat of respiratory health, whose function and regulation are context-specific.

Based on the known downregulation of SPLUNC1 by pathogens and inflammatory signals, we hypothesised that its sputum concentrations would decrease during AE, and that lower levels of SPLUNC1 would impair its host defence functions, leading to adverse clinical outcomes. Here, we show that SPLUNC1 decreases sharply as inflammation increases in AE, and that in stable patients lower SPLUNC1 levels portend an increased AE risk. SPLUNC1 downregulation occurs shortly after exposure to cytokines and proteases, suggesting that it could detect AE at early stages, reducing diagnostic uncertainty and informing proactive interventions to decrease AE impact on CF health [44–46].

Materials and methods

Definition of CF exacerbation

AE was defined as the emergence of four out of 12 signs or symptoms, prompting changes in therapy and initiation of antibiotics (modified from Fuchs' criteria [18]). These criteria included change in sinus congestion, sputum or haemoptysis; increased cough, dyspnoea, malaise, fatigue or lethargy; fever; hyporexia or weight loss; change in chest physical exam; or FEV_1 decrease >10% from a previous value [18]. Individuals not meeting AE criteria were characterised as "CF stable".

Study design

This was a two-centre, prospective study of CF participants during periods of clinical stability and AE. All patients received standard-of-care therapy and CFTR modulators when they became available. Our primary objective was to define an association between AE and sputum levels of SPLUNC1. Each participant provided a sputum sample and underwent spirometry within 24 h of sample collection. Participants were followed at quarterly outpatient clinic visits, or sooner when indicated, for up to 1 year (supplementary figure S1). Clinical information, sputum and spirometry data were collected at each visit.

Cohort characteristics

Discovery cohort

44 adults with confirmed CF diagnosis from the Yale Adult CF Program were recruited from 2014 to 2016 during 1) scheduled routine visits; 2) unscheduled visits in which they reported AE symptoms; and 3) on the first day of admission to the hospital for AE treatment. We organised study participants in two groups: 1) stable CF participants (CF stable): no new respiratory symptoms, presenting to clinic for scheduled follow-up and 2) AE participants (AE): diagnosed with AE (table 1). We also recruited 10 healthy controls to undergo sputum induction according to published protocols [47]. The study was approved by the Yale University institutional review board and informed consent was obtained from each participant.

TABLE 1 Demographic characteristics of the Yale Adult CF Program cohort (discovery cohort)

	HC	CF stable	CF AE
Participants	9	30	14
Age years	33.5±10.7 (27–45)	41.1±17.0 (20–79)	32.1±6.4 (23–43)
Sex			
Female	1 (11.1)	17 (56.7)	8 (57.1)
Male	8 (88.9)	13 (43.3)	6 (42.9)
Mutation background			
F508del/*F508del*	NA	11 (36.7)	8 (57.1)
F508del/other	NA	11 (36.7)	4 (28.6)
Other mutations	NA	8 (26.7)	2 (14.3)
FEV_1			
FEV_1 L	NA	2.2±0.7 (0.57–3.3)	1.9±0.7 (0.55–2.98)
FEV_1 %	NA	67.5±24.3 (12–121)	56.1±24.3 (12–89)
BMI kg·m^{-2}	NA	24.4±4.1 (17.6–35.8)	22.7±4 (17.6–35.8)
Exacerbations per year mean (range)	NA	1.9 (0–10)	3.7 (1–10)
CF comorbidities			
Pancreatic insufficiency	NA	25 (83.3)	14 (100)
CF-related diabetes	NA	11 (36.7)	9 (64.3)
Microbiology			
Pseudomonas aeruginosa colonisation	NA	12 (40)	9 (64.3)
CFTR modulators			
Ivacaftor	NA	2 (6.7)	0 (0)
Ivacaftor/lumacaftor	NA	6 (20)	7 (50)

Data are presented as n, mean±SD (range) or n (%), unless otherwise stated. HC: healthy controls; CF stable: cystic fibrosis participants without exacerbation; CF AE: CF participants with active pulmonary exacerbation; FEV_1: forced expiratory volume in 1 s; BMI: body mass index; CFTR: cystic fibrosis transmembrane conductance regulator; NA: not applicable.

Validation cohort

35 adult and paediatric participants with confirmed CF, previously enrolled in a prospective study of patients hospitalised for AE treatment at the University of Minnesota (UMN), were included [23]. All patients received standard-of-care therapy and each participant provided sputum samples and performed pulmonary function tests within 72 h of antibiotic initiation (table 2) [48].

Sputum collection and processing

CF participants expectorated sputum spontaneously for cultures and provided an additional study sample. Induced sputum samples were obtained from healthy controls by induction as reported previously [47, 49]. Sputum was diluted, filtered, centrifuged and processed as reported previously [50].

SPLUNC1 and cytokine ELISA

A direct SPLUNC1 ELISA was developed in our laboratory to measure SPLUNC1 in sputum (details in supplementary methods). Briefly, high-binding polystyrene ELISA plates (Corning, NY, USA; cat# 9018) were coated with sputum supernatants or recombinant human SPLUNC1 protein (rhSPLUNC1) as reference (Abnova, Taipei, Taiwan; cat# H00051297-P01). A polyclonal mouse anti-human SPLUNC1 IgG (MilliporeSigma, Burlington, MA, USA; cat# SAB1401687) was used as detection antibody and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, USA; cat# G21040) as secondary. Chromogenic tetramethylbenzidine substrate was applied (KPL, Gaithersburg, MD, USA; cat# 5120-0047-50-76-00) and reactions were measured at optical densities of 450 and 550 nm. The assay limits of detection were 1–20 000 ng·mL^{-1}. Mean±SD intra-assay variability was 5.18±1.28% and inter-assay variability was 18.44±12.95%.

Custom-made multiplexed cytokine ELISA assays were used to measure cytokine levels in sputum. Briefly, biotinylated capture antibodies for CXCL10, granulocyte colony-stimulating factor (G-CSF), interferon (IFN)-α2a, IFN-γ, IL-1β, IL-13, IL-29, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and TNF-α were combined with an assigned "linker" for each cytokine. The linker–antibody mix was then coated onto U-plex plates and incubated overnight according to manufacturer's specifications (U-Plex Biomarker Kit; Mesoscale Diagnostics (MSD), Rockville, MD, USA; cat# K15235N-1). The following day, recombinant human cytokines and sputum samples were

TABLE 2 Demographic characteristics of the University of Minnesota Cystic Fibrosis (CF) Center cohort (validation cohort)

	CF stable	CF AE
Participants	11	24
Age years	27.1±7.7 (14–40)	30±10.5 (13–57)
Sex		
Female	7 (63.6)	12 (50)
Male	4 (36.4)	12 (50)
Mutation background		
F508del/*F508del*	6 (54.5)	14 (58.3)
F508del/other	5 (45.5)	9 (37.5)
Other mutations	1 (9.1)	0 (0)
FEV_1		
FEV_1 L	2.4±0.6 (1.39–3.85)	1.7±0.6 (0.70–2.80)
FEV_1 %	67.3±12.1 (45.5–86.5)	45.3±14.1 (26–74)
BMI kg·m^{-2}	21.8±2.1 (18.1–24.6)	21.3±3.1 (13.1–25.8)
Exacerbations per year mean (range)	4.1 (1–12)	4.3 (1–10)
CF comorbidities		
Pancreatic insufficiency	11 (100)	23 (95.8)
CF-related diabetes	6 (54.5)	9 (37.5)
Microbiology		
Pseudomonas aeruginosa colonisation	5 (45.5)	9 (37.5)
CFTR modulators		
Ivacaftor	0 (0)	0 (0)
Ivacaftor/lumacaftor	0 (0)	0 (0)

Data are presented as n, mean±SD (range) or n (%), unless otherwise stated. CF stable: CF participants without exacerbation; CF AE: CF participants with active pulmonary exacerbation; FEV_1: forced expiratory volume in 1 s; BMI: body mass index; CFTR: cystic fibrosis transmembrane conductance regulator.

loaded onto U-Plex plates. Finally, detection antibodies for each cytokine were applied and Read Buffer T was added to each well to quantify the reaction. Plates were read on a Quickplex SQ 120 reader (MSD; cat# AI0AA-0) using MSD Discovery Workbench software version 4.0.

Western blot

Western blots were performed as reported previously, using human neutrophil elastase (hELA2), mouse monoclonal anti-hELA2 IgG (R&D Systems, Minneapolis, MN, USA; cat# MAB-91671-100) and mouse polyclonal anti-human SPLUNC1 IgG [28]. HRP-conjugated anti-mouse IgG (Invitrogen; cat# G21040) was used as secondary antibody. Membranes were developed using chemiluminescence and protein band densitometry was determined using ImageJ software version 1.7 (https://imagej.nih.gov/ij/index.html).

Sputum neutrophil elastase activity and SPLUNC1 degradation assays

Neutrophil elastase (NE) activity was determined using the 7-amino-4-methylcoumarin assay (Peptides International, Louisville, KY, USA; #MAA-3133), as described [28]. rhSPLUNC1 was incubated with recombinant human NE (rhNE; R&D Systems; cat#9167-SE-020) or *P. aeruginosa* elastase (LasB, a gift from Karen Agaronyan, Yale University School of Medicine, New Haven, CT, USA) at decreasing concentrations for 3 and 8 h. SPLUNC1 concentrations were measured by ELISA. Starting NE concentrations (1 μM) were selected based on previous sputum NE level measurements by our group [28]. There were no published data on airway levels of LasB during AE to inform dose selection. However, a dose capable of inhibiting host defence peptide expression and inducing cytokine expression had been reported previously (3.75 μM) [51]. Based on this, we chose a starting dose of 1 μM to define minimal LasB doses capable of regulating SPLUNC1.

Regulation of epithelial cytokine expression

Mouse tracheal epithelial cells (mTECs) were isolated from C57BL/6 mice and cultured at air–liquid interface as described previously [26]. mTECs were treated with recombinant murine IL-1β (Peprotech, Rocky Hill, NJ, USA; cat# 211-11b) or TNF-α (Peprotech; cat# 315-01A) at 10 ng·mL^{-1} for 24 h. NCI-H292 human airway epithelial cells were treated with recombinant human IL-1β (Gibco, Gaithersburg, MD, USA; cat# PHC0811) or TNF-α (R&D; cat# 210-TA-005) at 10 ng·mL^{-1}. Cellular

mRNA was extracted for quantitative (q)PCR, and qPCR assays were performed to quantify *SPLUNC1* transcriptional regulation as described previously [26].

Statistical analysis

Descriptive statistics were calculated for the entire participant population. Pearson or Spearman correlations for variables that were not normally distributed, were calculated between SPLUNC1 and clinical parameters. In order to select optimal thresholds to separate groups at higher AE risk, we developed receiver-operator curves (ROC) based on the distribution of SPLUNC1, IL-1β, TNF-α, G-CSF, IL-6 and IL-8 levels in the discovery cohort (supplementary figure S2). Using these thresholds, we applied statistical modelling (Mantel–Haenszel estimator) to predict AE-free intervals. AE-free intervals were defined as the time in days from sputum sampling in a stable patient to the time of the first AE after that visit. Finally, a Cox proportional hazards model was conducted with clinical parameters as covariates. A backward elimination strategy with a significance level to stay of 95% (α=0.05) was employed to achieve a parsimonious model. All statistical analyses were conducted using SAS 9.4 with a level of significance of 95% (α=0.05).

Details are provided in the supplementary methods.

Results

SPLUNC1 is decreased in the sputum of stable CF participants

SPLUNC1 levels ranged from 4.41 to 22.24 $\mu g \cdot mL^{-1}$ in the sputum of healthy controls. In stable CF participants, SPLUNC1 was significantly decreased, whereas total sputum protein was increased (figure 1a and b). To further define the inflammatory profile of stable CF participants, we measured sputum concentrations of cytokines previously reported to be increased in CF. Of these, IFN-α, IFN-γ, IL-1β, IL-8, IL-13 and TNF-α were significantly increased in CF compared to healthy controls (figure 1c). There were no differences in SPLUNC1 levels of stable participants according to severity of lung function impairment, *F508del* genotype, use of CFTR modulators or microbiology findings (supplementary tables S1–S4, supplementary figure S3a–d). These findings indicate that SPLUNC1 is abundant in sputum and decreased in stable CF participants.

SPLUNC1 decreases further during AE

We measured SPLUNC1 levels in sputum from stable and AE participants to determine if SPLUNC1 is a marker of AE. SPLUNC1 decreased sharply during AE in the discovery cohort (71.9% decrease) and in the validation cohort (38.6% decrease) (figure 2a). In contrast, FEV_1 did not decrease in the discovery cohort AE group, but was significantly lower in the validation cohort (figure 2b).

Mean SPLUNC1 levels of AE participants treated with oral antibiotics (AEO) and intravenous antibiotics (AEIV) in the UMN cohort were lower than stable CF levels. However, there was no difference in SPLUNC1 levels between AEO and AEIV (supplementary figure S4). The lack of difference between these treatment groups suggests that acute drops in SPLUNC1 occur during an AE regardless of its severity.

Next, we sought to define SPLUNC1 fluctuations during AE within the same individuals, relative to their stable-state reference value (individual-specific fluctuations). We compared SPLUNC1 and FEV_1 (%) in paired samples from the same participants, collected during stable and AE periods. SPLUNC1 decreased during AE in the majority of paired samples from both the Yale and UMN cohorts (figure 3a). In contrast, FEV_1 decreased during AE in the majority of UMN samples, but not in those form the Yale cohort (figure 3b). These findings indicate that while SPLUNC1 is consistently decreased during AE, FEV_1 changes during AE vary across cohorts.

Low SPLUNC1 levels predict AE risk in stable CF participants

To determine if SPLUNC1 is a predictor of AE risk, we first performed a Mantel–Haenszel survival estimator analysis for AE-free time. We separated the cohorts into high-/low-SPLUNC1 groups based on a concentration threshold defined by ROC analysis comparing AE and stable patients (supplementary methods, supplementary figure S2). In stable CF participants, the SPLUNC1-low group had a median AE-free time of 43.5 days, compared to 150 days in the SPLUNC1-high group; this relationship was preserved in a subgroup analysis of patients with FEV_1 >40% predicted (supplementary figure S5). This suggests that higher SPLUNC1 levels are associated with longer AE-free intervals independently of stable-state FEV_1.

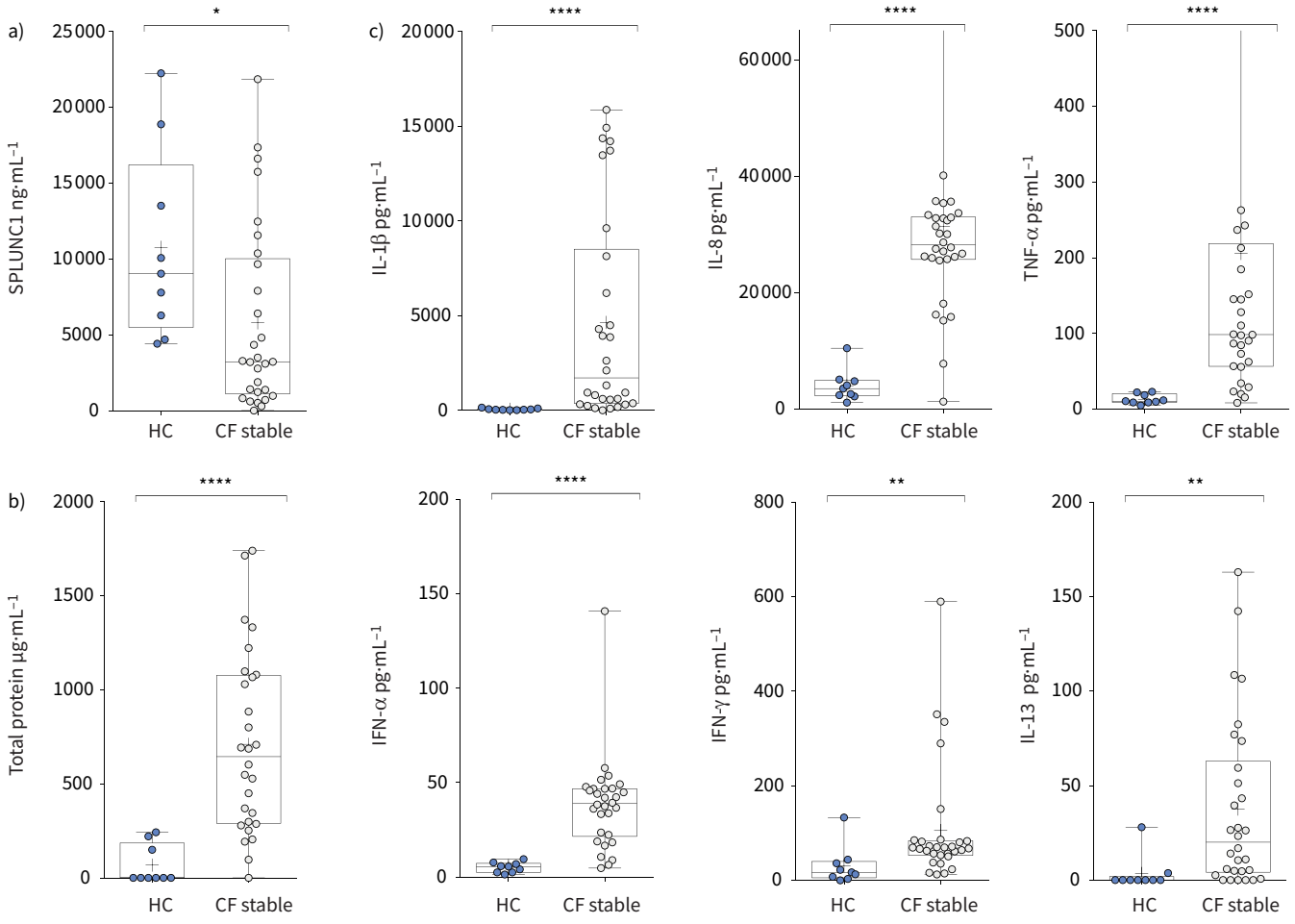

FIGURE 1 Short palate lung nasal epithelium clone 1 (SPLUNC1) is decreased in the sputum of stable cystic fibrosis (CF) patients. a) SPLUNC1 levels (ELISA) in sputum samples from the Yale cohort of adult CF patients without respiratory symptoms (CF stable) and healthy controls (HC). b) Total protein in sputum (bicinchoninic acid assay) from the same patients. c) Inflammatory cytokine levels (ELISA) in sputum from the same patients. Additional cytokines tested without significant difference: CXCL10, granulocyte colony-stimulating factor (G-CSF), interferon (IFN)-λ, interuleukin (IL)-6, IL-13, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)1α. CF samples were obtained by voluntary expectoration during clinical assessment; HC samples were obtained by sputum induction with nebulised normal saline solution. Data are presented as mean (+), median and range. TNF: tumour necrosis factor. *: p<0.05; **: p<0.01; ****: p<0.0001, Mann–Whitney test with Bonferroni correction.

Next, we performed Cox-proportional hazards modelling to assess the likelihood of AE while adjusting for demographics, CFTR genotype, CF-related comorbidities, microbiology and lung function. In the short-term (60 days), participants in the SPLUNC1-low group had a significantly increased risk of AE (hazard ratio (HR) 11.49, p=0.003; figure 4a), which persisted upon long-term follow-up at 1 year (HR 3.21, p=0.013; figure 4b).

In order to compare SPLUNC1 to previously reported biomarkers as predictors of AE, we defined ROC thresholds and AE-free time for G-CSF, IL-1β, IL-6, IL-8 and TNF-α (supplementary figure S2). In a similar multivariate proportional hazards model, cytokine high/low groups based on these markers did not show an increased hazard ratio of AE at 60 days, and only high IL-1β and TNF-α were associated with an increased AE risk at 1 year of follow-up (HR 3.90 and 3.46, respectively, p<0.05; supplementary figure S6). These findings suggest that SPLUNC1 is a better predictor of AE risk in the short and long term than previously reported sputum AE markers.

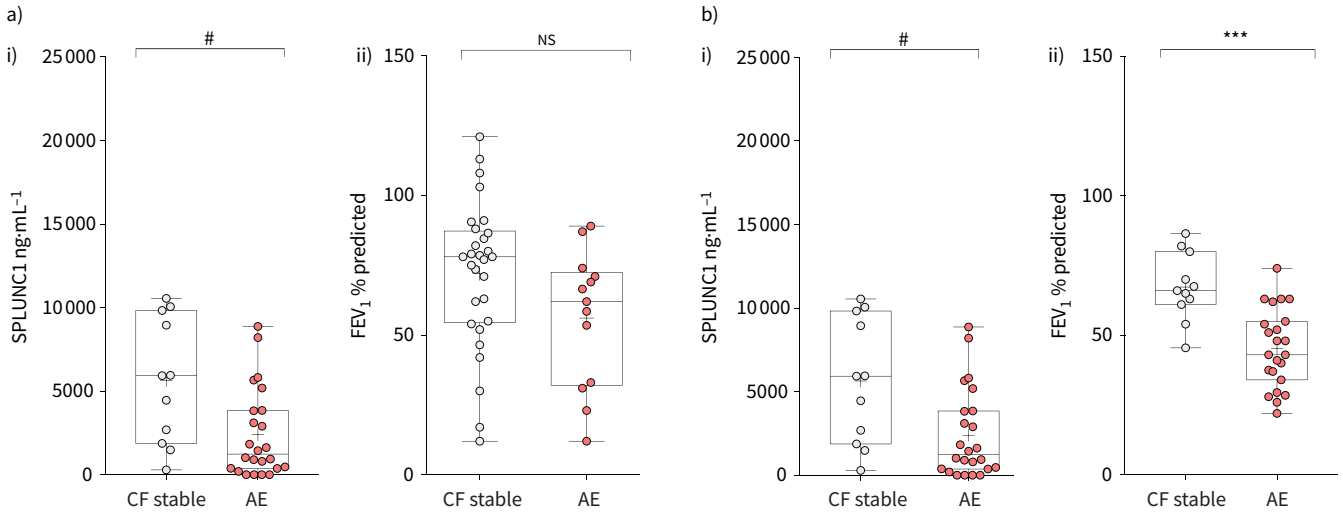

FIGURE 2 Short palate lung nasal epithelium clone 1 (SPLUNC1) is decreased during acute cystic fibrosis (CF) exacerbations (AE). Sputum SPLUNC1 and forced expiratory volume in 1 s (FEV_1) from two clinical cohorts including a) adult (Yale University, n=43) and b) mixed adult/paediatric (University of Minnesota, n=35) CF patients. Samples were obtained by voluntary expectoration during clinical assessment. i) SPLUNC1 quantified by ELISA; ii) FEV_1 obtained by spirometry during clinical assessment. CF stable: no symptoms of AE, no antibiotic treatment; AE: acute CF exacerbation, symptoms of AE and ongoing antibiotic therapy; NS: nonsignificant. Data are presented as mean (+), median and range. #: p<0.005; ***: p<0.001, Mann–Whitney test.

Human and bacterial elastases found in CF sputum degrade SPLUNC1

Our group and others have shown that NE degrades SPLUNC1, and that NE inhibitor sivelestat only partially prevents SPLUNC1 degradation by CF sputum [27, 28]. In order to understand the role of NE and bacterial elastase in decreasing SPLUNC1 during AE, we incubated rhSPLUNC1 with recombinant human neutrophil elastase (NE) or LasB at increasing concentrations for 3 and 8 h. Both elastases induced a concentration-dependent decrease in full-length SPLUNC1 (figure 5a and b). Next, we quantified NE concentrations in healthy control and CF sputum during stable and AE periods. NE was increased overall in CF, but it did not increase significantly from stable levels during AE (figure 5c). Finally, to define individual-specific NE and SPLUNC1 changes, we performed Western blots of healthy control and CF sputum, probing for NE, followed by re-probing for SPLUNC1. Although NE was increased in CF relative to healthy controls, NE levels were not different between stable and AE states (figure 5d).

To determine if NE activity, rather than concentration, increased during AE we measured NE-specific fluorescent cleavage products. When incubated with NE, CF sputum had much higher NE activity than healthy control sputum; however, there was no difference between stable and AE participants (figure 5e).

Sputum cytokines IL-1β and TNF-α are increased during AE

To further define the AE inflammatory profile of CF participants, we measured concentrations of 13 cytokines in stable and AE samples. Only IL-1β and TNF-α were significantly increased during AE (figure 6a). In addition, we sought to define a relationship between SPLUNC1 and cytokine levels in sputum during stable and AE states using Pearson's correlation. In stable and AE groups from both cohorts, IL-1β levels inversely correlated with SPLUNC1 (supplementary figure S7), while CXCL10, G-CSF, IFN-γ, IL-6, MCP1, MIP-1α and TNF-α did not consistently correlate with SPLUNC1 across cohorts (not shown).

In order to determine whether increased IL-1β and TNF-α contributed to decreased SPLUNC1 during AE, we treated mTEC and a human airway epithelial cell line with these cytokines and measured *SPLUNC1* mRNA expression. At concentrations encountered in AE sputum, both IL-1β and TNF-α decreased *SPLUNC1* expression by airway epithelial cells (figure 6b). Together with our observations from NE and LasB experiments, these findings suggest that during AE, SPLUNC1 is decreased through protein degradation and cytokine-driven transcriptional downregulation.

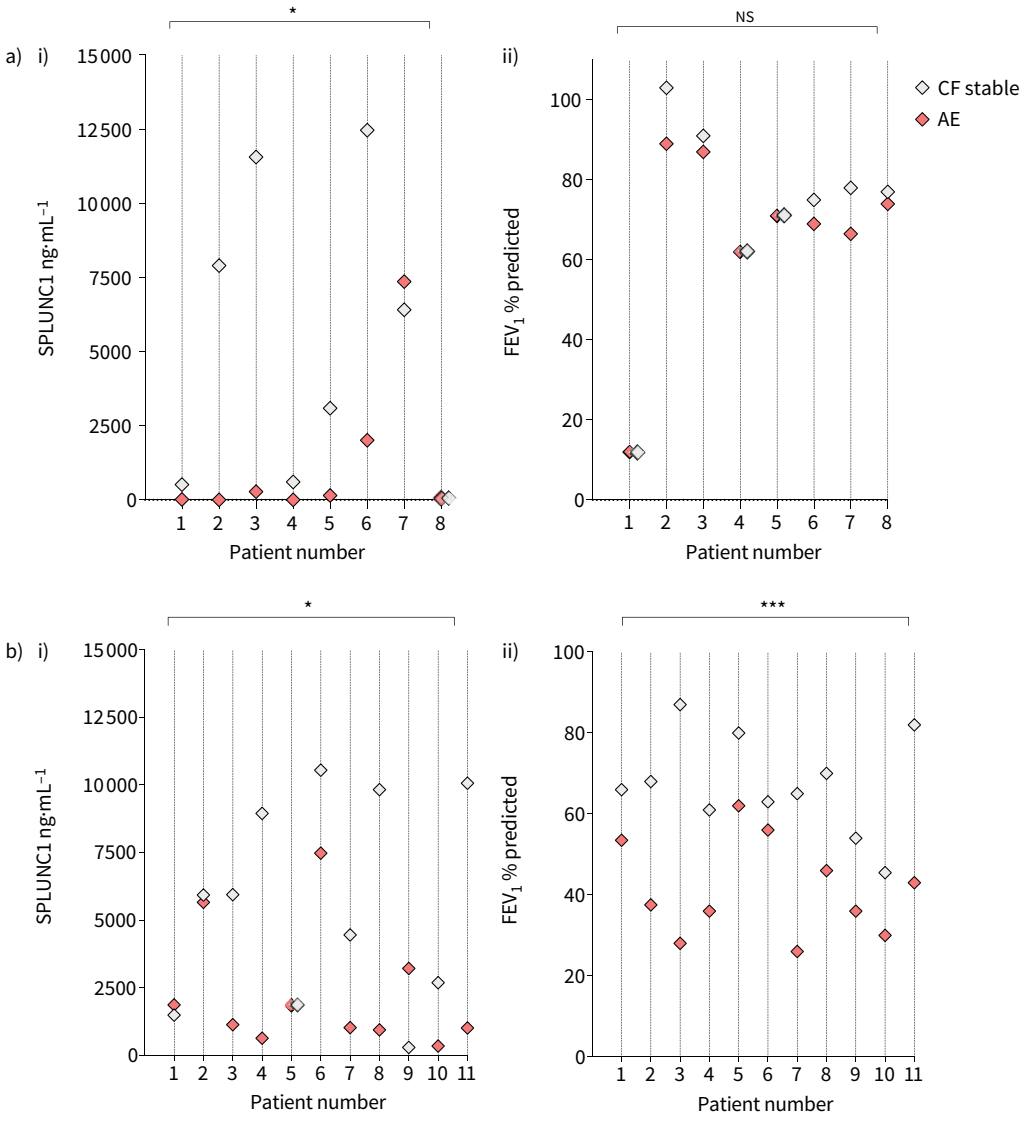

FIGURE 3 Individual-specific short palate lung nasal epithelium clone 1 (SPLUNC1) and forced expiratory volume in 1 s (FEV_1) decreases during acute cystic fibrosis (CF) exacerbations (AE). Samples from two clinical cohorts including a) adult (Yale University, n=8) and b) mixed adult/paediatric CF patients (University of Minnesota, n=11). i) Paired SPLUNC1 levels in sputum samples from the same individual with and without AE (ELISA); ii) paired FEV_1 measurements from the same individual with and without AE obtained by spirometry during clinical assessment. Each vertical line and number represent a single patient who provided one stable and one AE sample. When values were the same, these were represented by two overlapping diamonds along the patient's line. CF stable: no symptoms of AE, no antibiotic treatment; AE: acute CF exacerbation, symptoms of AE and ongoing antibiotic therapy; NS: nonsignificant. *: $p<0.05$, ***: $p=0.0001$, Wilcoxon matched-pairs signed-rank test.

Discussion

AEs contribute to increased morbidity in CF and treatment delays are associated with poor FEV_1 recovery and impaired treatment responses [5–9, 15–17, 52, 53]. Yet, few biomarkers are clinically available to guide early AE interventions in order to minimise hospitalisations and improve quality of life [44–46]. Here, we describe a novel role for SPLUNC1 as an AE biomarker and predictor that could support clinical decision-making to improve AE outcomes.

The key finding of our study is that among CF participants, SPLUNC1 levels were considerably lower during AE compared to stable state. We propose that the mechanism for SPLUNC1 decreases in CF is

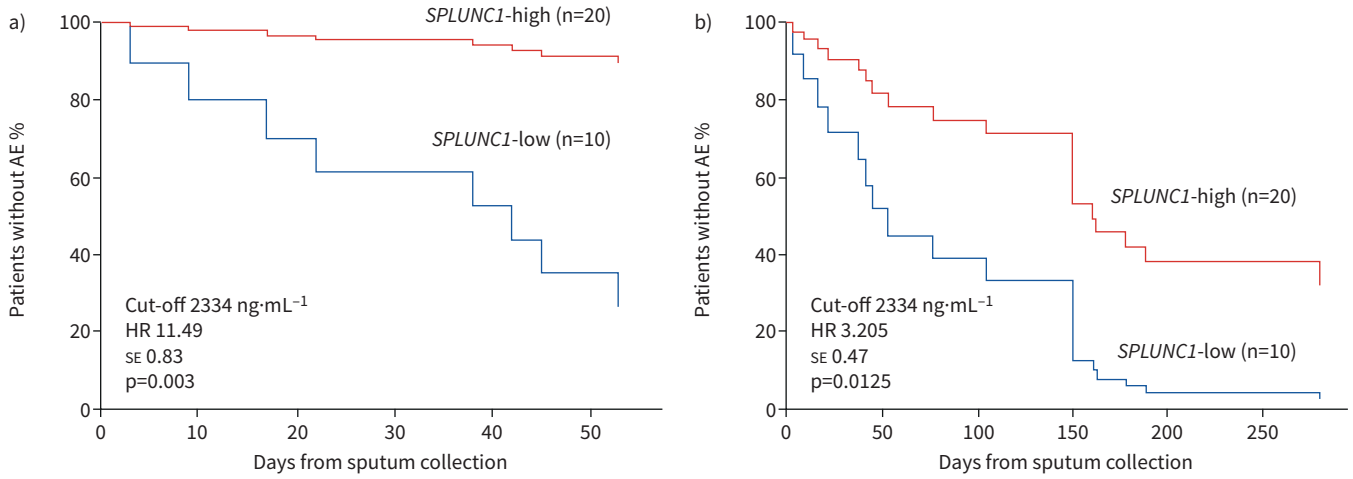

FIGURE 4 Short palate lung nasal epithelium clone 1 (SPLUNC1) predicts acute pulmonary exacerbation (AE)-free time. a) AE-free time in stable cystic fibrosis (CF) patients separated into SPLUNC1-high and SPLUNC1-low groups over a 60-day follow-up period. SPLUNC1-high and -low groups were defined according to sputum concentration thresholds obtained from receiver-operator curves separating CF stable and AE levels (supplementary figure S4). AE-free time was defined as the number of days from sputum collection in stable patients until the date of their next AE. b) AE-free time in stable CF patients separated into SPLUNC1-high and -low groups over a 365-day follow-up period. Cox proportional hazards model used to calculate AE-free intervals and adjust for age, sex, body mass index, forced expiratory volume in 1 s, number of *F508del* mutations, presence of CF-related diabetes or pancreatic insufficiency, use of cystic fibrosis transmembrane conductance regulator modulators and microbiology for *Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, *Haemophilus parainfluenzae*, methicillin-sensitive *Staphylococcus aureus* and methicillin-resistant *S. aureus*.

multifactorial, with contributions from protease degradation and gene expression downregulation by inflammatory cytokines. Prospectively, stable patients with low SPLUNC1 had an increased likelihood of AE at 60 days and 1 year. These findings suggest that SPLUNC1 levels could inform the diagnosis and clinical management of AE in the short and long term.

By the time symptoms develop or FEV_1 declines, airway inflammation and damage may already be underway [14, 15]. In the face of a suspected AE with incomplete clinical criteria and normal spirometry, a low SPLUNC1 level would support a decision to increase airway clearance, adjust monitoring or initiate pharmacological interventions when appropriate. In asymptomatic stable patients, low SPLUNC1 levels could also prompt immediate or long-term changes in clinical management.

Although symptom and spirometry monitoring at home increase AE detection, they do not prevent FEV_1 decline, possibly because of the delayed nature of FEV_1 changes in response to airway inflammation [54]. The application of clinical biomarkers is even more challenging in children, where FEV_1 and cytokine abnormalities are inconsistently detected until adolescence, despite early evidence of structural lung disease [44, 55–57]. SPLUNC1 measurements at home could detect subtle inflammatory changes that complement symptom and spirometry monitoring.

Previous studies examined the correlation between sputum biomarkers, infection, inflammation, and lung function decline in CF [25, 58–67]. While some defined novel AE biomarkers [25, 68–72], to our knowledge, ours is the first study to compare a broad panel of markers in AE and stable state that includes adults and children.

Daily changes in inflammatory signals or pathogen exposures may cause small fluctuations; however, SPLUNC1 levels drop sharply during AE. We previously showed that lipopolysaccharide (LPS) and IFN-γ have tonic suppressive effects on SPLUNC1 at baseline [26]. However, high-dose LPS and IFN-γ exposures decrease epithelial *SPLUNC1* expression drastically, indicating a dose-dependent response. SPLUNC1's tight regulation suggests that it is an ideal biomarker to detect early and subtle changes in lung homeostasis [26].

SPLUNC1 has host protective functions relevant to CF, including regulation of airway surface liquid, antimicrobial properties and immunomodulatory effects [73–79]. Therefore, SPLUNC1 decreases in CF

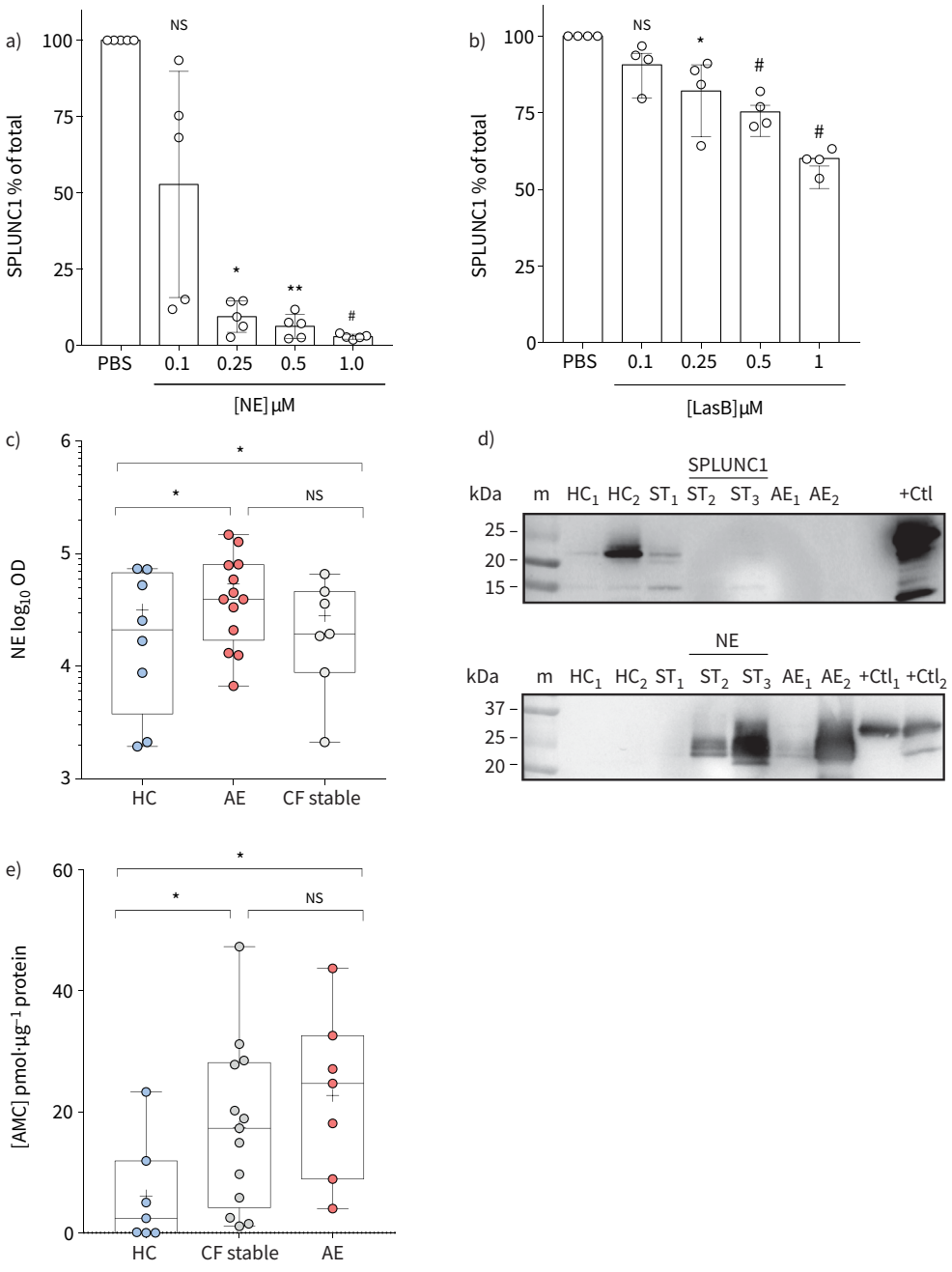

FIGURE 5 Elastase concentration and activity are increased in cystic fibrosis (CF). a) Short palate lung nasal epithelium clone 1 (SPLUNC1) densitometry showing degradation by human neutrophil elastase (NE) relative to PBS control, at specified concentrations over 3 h at 37°C. b) SPLUNC1 densitometry showing degradation by elastase B (LasB) from *Pseudomonas aeruginosa* relative to PBS control at specified concentrations over 8 h at 37°C. n=4–5; two individual experiments. c) NE densitometry in sputum from healthy controls (HC), CF patients with no symptoms of acute CF exacerbation (AE), no antibiotic treatment (CF stable) and patients with acute CF exacerbation, symptoms of AE and ongoing antibiotic therapy (AE) assessed by Western blot. d) Representative Western blot showing endogenous expression of SPLUNC1 (20–25 kDa) and NE (25–30 kDa) in HC and CF sputum samples from the same individuals. Membranes were probed for NE prior to stripping and re-probing for SPLUNC1. e) NE activity in CF sputum: the 7-amino-4-methylcoumarin (AMC) formation from florigenic NE substrate MAA-3133 following 6 h incubation with healthy control (HC), CF stable and AE sputum

at 37°C. Data are presented as mean (+), median and range. NS: nonsignificant; OD: optic density; AE: acute CF exacerbation, symptoms of AE and ongoing antibiotic therapy; CF stable: no symptoms of AE, no antibiotic treatment; m: marker; ST: stable CF; +Ctl: recombinant protein positive control. *: p<0.05; **: p<0.01; #: p<0.005, Mann–Whitney test.

may not only be a marker, but also a contributor to pathogenesis during AE and disease progression. Decreased SPLUNC1 in CF may impair mucociliary clearance and facilitate bacterial colonisation, leading to tissue injury and exacerbated inflammation. In fact, some SPLUNC1-deficient animal models have shown increased susceptibility to infectious and non-infectious inflammation [80, 81]. In our study, we observed increased IL-1β and TNF-α during AE, but only IL-1β inversely correlated with SPLUNC1. This correlation may reflect the transcriptional effects of higher relative concentrations of IL-1β during AE when compared to TNF-α (figure 6a). Furthermore, others have shown that IL-1β may have a more potent neutrophil recruitment effect than TNF-α [82]. Thus, the increased concentrations of IL-1β may enhance neutrophil recruitment that in turn increases SPLUNC1 degradation, strengthening the inverse correlation between SPLUNC1 and IL-1β. Our data showing increased NE levels and activity, although not different between AE and stable state, suggest that SPLUNC1 cleavage at functional sites by NE (and other proteases during AE) may disrupt its host defence functions, worsening inflammation and accelerating lung disease [34, 83].

Our study has some limitations. First, our study has a small sample size and predicts AE risk based on cross-sectional data. However, our findings show it was adequately powered to demonstrate differences in key observations, confirmed on a validation cohort. In future, we would favour serial SPLUNC1 measurements to establish stable-state baselines that reflect day-to-day fluctuations. Second, there is no distinct SPLUNC1 level that separates healthy controls from CF, or stable- from AE-state in all participants. The overlap of SPLUNC1 levels among some healthy controls and CF patients may be in part explained by changes in inflammation and environmental exposures between sputum samplings. However, our study highlights the value of individual biomarker variability in characterising disease states. Third, we used *i.v.* or oral therapy as surrogates for disease severity. We appreciate that choice of therapy route is

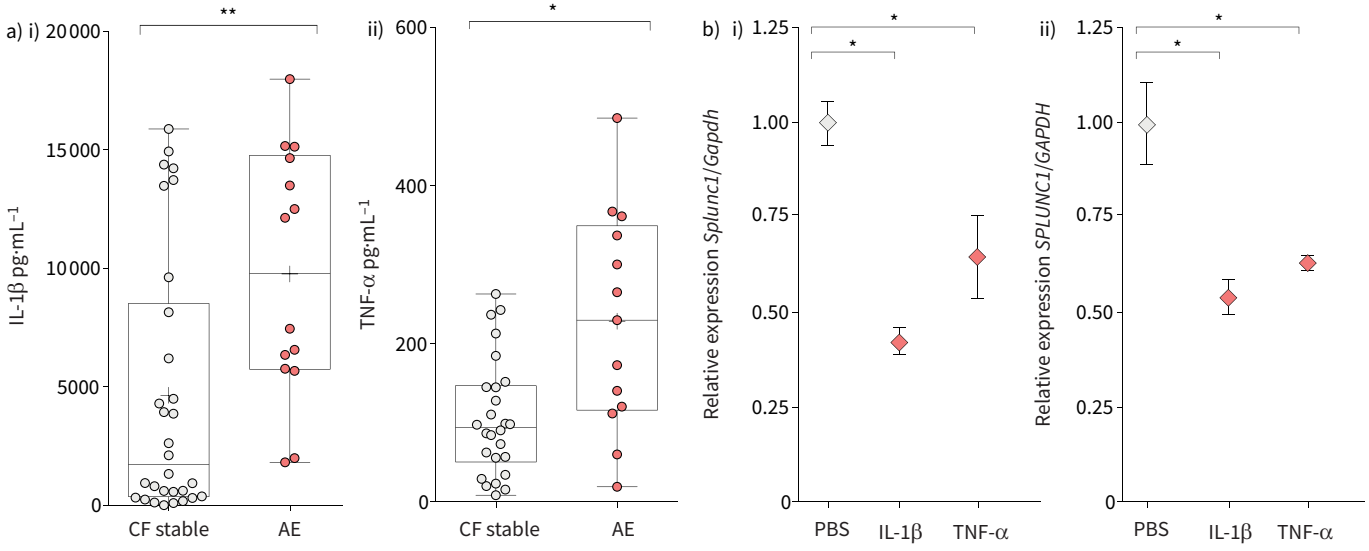

FIGURE 6 Cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α increase during acute cystic fibrosis (CF) exacerbation (AE) and downregulate short palate lung nasal epithelium clone 1 (SPLUNC1) expression. a) Inflammatory cytokine levels in sputum from adult CF patients without respiratory symptoms (CF stable) and with acute CF exacerbation (AE) i) IL-1β, ii) TNF-α. Additional cytokines tested without significant difference: CXCL10, granulocyte colony-stimulating factor (G-CSF), interferon (IFN)-α2, interferon (IFN)-γ, IFNλ, IL-6, IL-8, IL-13, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α. b) Relative *SPLUNC1* mRNA expression in i) mouse tracheal epithelial cells (mouse epithelium) grown at air–liquid interface and ii) the NCI-H292 human airway epithelial cell line (human epithelium) treated with recombinant TNF-α and IL-1β (10 ng·mL^{-1}) for 24 h (two-way ANOVA). Data are presented as mean (+), median and range. mRNA expression quantified by qPCR. Represents two experiments. *: p<0.05; **: p<0.01, Mann–Whitney test with Bonferroni correction.

based on many factors, including access to care, AE complications and severity of FEV_1 decline [84]. We decided to present these data to show that SPLUNC1 can be used as a marker in AE of any severity and regardless of factors driving therapeutic decisions. Finally, our cohorts had a higher prevalence of advanced lung disease and *CFTR* genotypes linked to severe disease. We addressed this in our multivariate models by using a backwards elimination strategy to confirm that the predictive ability of SPLUNC1 was not affected by *CFTR* genotype. Larger prospective studies are needed to replicate our findings in subcohorts that reflect specific *CFTR* genotypes, comorbidities and the impact of novel CFTR modulator combinations.

In the age of highly effective CFTR modulator therapy, we look forward to rising life expectancy and quality of life [4]. We hope that measurements of noninvasive, airway-relevant biomarkers such as SPLUNC1 will become a resource to guide acute management of respiratory complications and inform our partnership with CF patients for the betterment of their long-term health.

Acknowledgements: We thank our patients, the medical staff at the Yale Adult Cystic Fibrosis Program and Jonathan Koff, Director of the Adult CF Program, for their support and contributions to this project. We also thank Mehmet Kesimer from the University of North Carolina at Chapel Hill for his thoughtful review and contributions in the development of this manuscript.

Author contributions: C.J. Britto, N. Niu, S. Khanal, J. Zielonka, L. Sharma and C.S. Dela Cruz planned the project, designed and performed experiments, analysed the data, and wrote the manuscript. T. Laguna and M. Nunez contributed to the design and analysis of clinical data, and provided access to the UMN cohort samples. M. Webster and R. Tarran performed and analysed experiments related to NE densitometry and activity in sputum. G. Chupp generated the sputum collection protocol for the Yale cohort and facilitated access to banked human samples. L. Cohn, M. Sauler, J.L. Gomez, M. Egan and G. Chupp contributed to the design and analysis of all experiments, and the final manuscript. M.D. Slade provided biostatistics support and analysed all experiments. All authors reviewed, revised and approved the manuscript for submission.

Conflict of interest: S. Khanal has nothing to disclose. M. Webster has nothing to disclose. N. Niu has nothing to disclose. J. Zielonka has nothing to disclose. M. Nunez has nothing to disclose. G. Chupp reports other (advisor, clinical trial investigator, speaker) from Genentech, AstraZeneca and Sanofi-Regeneron, other (advisor, clinical trial investigator) from GSK, TEVA, Boehringer Ingelheim and Circassia, outside the submitted work. M.D. Slade has nothing to disclose. L. Cohn reports other (lectures) from Genentech, other (advisory board work) from Novartis, AstraZeneca, GlaxoSmithKline, Regeneron, Pieris and Sanofi, outside the submitted work. M. Sauler has nothing to disclose. J.L. Gomez has nothing to disclose. R. Tarran reports other (founder and equity) from Eldec Pharmaceuticals, outside the submitted work; and has a patent Peptide inhibitors of Ca^{2+} channels pending, a patent Peptide inhibitors of sodium channels with royalties paid, and a patent Regulation of sodium channels by PLUNC proteins with royalties paid. L. Sharma has nothing to disclose. C.S. Dela Cruz has nothing to disclose. M. Egan has nothing to disclose. T. Laguna reports grants from National Institutes of Health and Cystic Fibrosis Foundation, other (advisory board honorarium) from Vertex, outside the submitted work. C.J. Britto has nothing to disclose.

Support statement: This work was supported by NIH R01-HL081160 and R21-AI083475 (L. Cohn), NIH T32-HL007778, NIH/NHLBI K01-HL125514-01, and Cystic Fibrosis Foundation's Fifth Year Clinical Fellowship and Pilot and Feasibility Award (C.J. Britto), American Thoracic Society Foundation Unrestricted Research Award (C.J. Britto), CF Foundation (R. Tarran); UK CF Trust (R. Tarran). Funding information for this article has been deposited with the Crossref Funder Registry.

References

1 Cystic Fibrosis Foundation Patient Registry. 2018 Annual Data Report. Bethesda, Cystic Fibrosis Foundation, 2019.
2 Rowe SM, Miller S, Sorscher EJ. Cystic fibrosis. *N Engl J Med* 2005; 352: 1992–2001.
3 Welsh MJ, Ramsey BW, Accurso F, *et al*. Part 21: membrane transport disorders. Cystic fibrosis. *In:* Beaudet AL, Scriver CR, Sly WS, *et al.*, eds. The metabolic and molecular basis of inherited disease. New York, McGraw-Hill, 2001; 5121–5189.
4 Cystic Fibrosis Foundation Patient Registry. 2019 Annual Data Report. Bethesda, Cystic Fibrosis Foundation, 2020.
5 Flight WG, Bright-Thomas RJ, Sarran C, *et al.* The effect of the weather on pulmonary exacerbations and viral infections among adults with cystic fibrosis. *Int J Biometeorol* 2014; 58: 1845–1851.
6 Flight WG, Bright-Thomas RJ, Tilston P, *et al.* Incidence and clinical impact of respiratory viruses in adults with cystic fibrosis. *Thorax* 2014; 69: 247–253.

7 Goss C H, Burns JL. Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. *Thorax* 2007; 62: 360–367.
8 Pribble CG, Black PG, Bosso JA, *et al.* Clinical manifestations of exacerbations of cystic fibrosis associated with nonbacterial infections. *J Pediatr* 1990; 117: 200–204.
9 Wark PA, Tooze M, Cheese L, *et al.* Viral infections trigger exacerbations of cystic fibrosis in adults and children. *Eur Respir J* 2012; 40: 510–512.
10 Etherington C, Naseer R, Conway SP, *et al.* The role of respiratory viruses in adult patients with cystic fibrosis receiving intravenous antibiotics for a pulmonary exacerbation. *J Cyst Fibros* 2014; 13: 49–55.
11 Whelan FJ, Surette MG. Clinical insights into pulmonary exacerbations in cystic fibrosis from the microbiome. What are we missing? *Ann Am Thorac Soc* 2015; 12: Suppl. 2, S207–S211.
12 Parkins MD, Floto RA. Emerging bacterial pathogens and changing concepts of bacterial pathogenesis in cystic fibrosis. *J Cyst Fibros* 2015; 14: 293–304.
13 van Ewijk BE, van der Zalm MM, Wolfs TF, *et al.* Viral respiratory infections in cystic fibrosis. *J Cyst Fibros* 2005; 4: Suppl. 2, 31–36.
14 Liou TG, Adler FR, Fitzsimmons SC, *et al.* Predictive 5-year survivorship model of cystic fibrosis. *Am J Epidemiol* 2001; 153: 345–352.
15 de Boer K, Vandemheen KL, Tullis E, *et al.* Exacerbation frequency and clinical outcomes in adult patients with cystic fibrosis. *Thorax* 2011; 66: 680–685.
16 Sanders DB, Hoffman LR, Emerson J, *et al.* Return of FEV_1 after pulmonary exacerbation in children with cystic fibrosis. *Pediatr Pulmonol* 2010; 45: 127–134.
17 Smith AL, Fiel SB, Mayer-Hamblett N, *et al.* Susceptibility testing of *Pseudomonas aeruginosa* isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. *Chest* 2003; 123: 1495–1502.
18 Fuchs HJ, Borowitz DS, Christiansen DH, *et al.* Effect of aerosolised recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. *N Engl J Med* 1994; 331: 637–642.
19 Stanford GE, Dave K, Simmonds NJ. Pulmonary exacerbations in adults with cystic fibrosis: a grown-up issue in a changing cystic fibrosis landscape. *Chest* 2021; 159: 93–102.
20 Kraynack NC, Gothard MD, Falletta LM, *et al.* Approach to treating cystic fibrosis pulmonary exacerbations varies widely across US CF care centers. *Pediatr Pulmonol* 2011; 46: 870–881.
21 West NE, Beckett VV, Jain R, *et al.*. Standardized Treatment of Pulmonary Exacerbations (STOP) study: physician treatment practices and outcomes for individuals with cystic fibrosis with pulmonary exacerbations. *J Cyst Fibros* 2017; 16: 600–606.
22 Harun SN, Wainwright C, Klein K, *et al.* A systematic review of studies examining the rate of lung function decline in patients with cystic fibrosis. *Paediatr Respir Rev* 2016; 20: 55–66.
23 Laguna TA, Williams CB, Brandy, KR, *et al.* Sputum club cell protein concentration is associated with pulmonary exacerbation in cystic fibrosis. *J Cyst Fibros* 2015; 14: 334–340.
24 Sagel SD, Kapsner R, Osberg I, *et al.* Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. *Am J Respir Crit Care Med* 2001; 164: 1425–1431.
25 Sagel SD, Wagner BD, Anthony MM, *et al.* Sputum biomarkers of inflammation and lung function decline in children with cystic fibrosis. *Am J Respir Crit Care Med* 2012; 186: 857–865.
26 Britto CJ, Liu Q, Curran DR, *et al.* Short palate, lung, and nasal epithelial clone-1 is a tightly regulated airway sensor in innate and adaptive immunity. *Am J Respir Cell Mol Biol* 2013; 48: 717–724.
27 Jiang D, Wenzel SE, Wu, Q, *et al.* Human neutrophil elastase degrades SPLUNC1 and impairs airway epithelial defense against bacteria. *PLoS One* 2013; 8: e64689.
28 Webster MJ, Reidel B, Tan CD, *et al.* SPLUNC1 degradation by the cystic fibrosis mucosal environment drives airway surface liquid dehydration. *Eur Respir J* 2018; 52: 1800668.
29 Bingle CD, Bingle L. Characterisation of the human *plunc* gene, a gene product with an upper airways and nasopharyngeal restricted expression pattern. *Biochim Biophys Acta* 2000; 1493: 363–367.
30 Bartlett JA, Hicks BJ, Schlomann JM, *et al.* PLUNC is a secreted product of neutrophil granules. *J Leukoc Biol* 2008; 83: 1201–1206.
31 Bingle L, Bingle CD. Distribution of human PLUNC/BPI fold-containing (BPIF) proteins. *Biochem Soc Trans* 2011; 39: 1023–1027.
32 Erickson NA, Dietert K, Enders, J, *et al.* Soluble mucus component CLCA1 modulates expression of leukotactic cytokines and BPIFA1 in murine alveolar macrophages but not in bone marrow-derived macrophages. *Histochem Cell Biol* 2018; 149: 619–633.
33 Britto CJ, Cohn L. Bactericidal/permeability-increasing protein fold-containing family member A1 in airway host protection and respiratory disease. *Am J Respir Cell Mol Biol* 2015; 52: 525–534.
34 Walton WG, Ahmad S, Little MS, *et al.* Structural features essential to the antimicrobial functions of human SPLUNC1. *Biochemistry* 2016; 55: 2979–2991.

35 Schaefer N, Li X, Seibold, MA, *et al.* The effect of BPIFA1/SPLUNC1 genetic variation on its expression and function in asthmatic airway epithelium. *JCI Insight* 2019; 4: e127237.
36 Boon K, Bailey NW, Yang, J, *et al.* Molecular phenotypes distinguish patients with relatively stable from progressive idiopathic pulmonary fibrosis (IPF). *PLoS One* 2009; 4: e5134.
37 Wu T, Huang J, Moore, PJ, *et al.* Identification of BPIFA1/SPLUNC1 as an epithelium-derived smooth muscle relaxing factor. *Nat Commun* 2017; 8: 14118.
38 Moore PJ, Reidel B, Ghosh A, *et al.* Cigarette smoke modifies and inactivates SPLUNC1, leading to airway dehydration. *FASEB J* 2018; 32: 6559–6574.
39 De Smet EG, Seys LJ, Verhamme FM, *et al.* Association of innate defense proteins BPIFA1 and BPIFB1 with disease severity in COPD. *Int J Chron Obstruct Pulmon Dis* 2017; 13: 11–27.
40 Bingle L, Barnes FA, Cross SS, *et al.* Differential epithelial expression of the putative innate immune molecule SPLUNC1 in cystic fibrosis. *Respir Res* 2007; 8: 79.
41 Saferali A, Obeidat M, Bérubé J-C, *et al.* Polymorphisms associated with expression of BPIFA1/BPIFB1 and lung disease severity in cystic fibrosis. *Am J Respir Cell Mol Biol* 2015; 53: 607–614.
42 Saferali A, Tang AC, Strug LJ, *et al.* Immunomodulatory function of the cystic fibrosis modifier gene BPIFA1. *PLoS One* 2020; 15: e0227067.
43 Bingle L, Cross SS, High AS, *et al.* SPLUNC1 (PLUNC) is expressed in glandular tissues of the respiratory tract and in lung tumours with a glandular phenotype. *J Pathol* 2005; 205: 491–497.
44 Ranganathan SC, Hall GL, Sly PD, *et al.* Early lung disease in infants and preschool children with cystic fibrosis. what have we learned and what should we do about it? *Am J Respir Crit Care Med* 2017; 195: 1567–1575.
45 Britto MT, Kotagal UR, Hornung, RW, *et al.* Impact of recent pulmonary exacerbations on quality of life in patients with cystic fibrosis. *Chest* 2002; 121: 64–72.
46 Bhatt JM. Treatment of pulmonary exacerbations in cystic fibrosis. *Eur Respir Rev* 2013; 22: 205–216.
47 Yan X, Chu JH, Gomez J, *et al.* Noninvasive analysis of the sputum transcriptome discriminates clinical phenotypes of asthma. *Am J Respir Crit Care Med* 2015; 191: 1116–1125.
48 Laguna TA, Wagner BD, Luckey HK, *et al.* Sputum desmosine during hospital admission for pulmonary exacerbation in cystic fibrosis. *Chest* 2009; 136: 1561–1568.
49 Esther CR Jr, Peden DB, Alexis NE, *et al.* Airway purinergic responses in healthy, atopic nonasthmatic, and atopic asthmatic subjects exposed to ozone. *Inhal Toxicol* 2011; 23: 324–330.
50 Yao Y, Welp T, Liu Q, *et al.* Multiparameter single cell profiling of airway inflammatory cells. *Cytometry B Clin Cytom* 2017; 92: 12–20.
51 Saint-Criq V, Villeret B, Bastaert F, *et al. Pseudomonas aeruginosa* LasB protease impairs innate immunity in mice and humans by targeting a lung epithelial cystic fibrosis transmembrane regulator-IL-6-antimicrobial-repair pathway. *Thorax* 2018; 73: 49–61.
52 Aaron SD, Ramotar K, Ferris W, *et al.* Adult cystic fibrosis exacerbations and new strains of *Pseudomonas aeruginosa*. *Am J Respir Crit Care Med* 2004; 169: 811–815.
53 Wood RE, Leigh MW. What is a "pulmonary exacerbation" in cystic fibrosis? *J Pediatr* 1987; 111: 841–842.
54 Lechtzin N, Mayer-Hamblett N, West NE, *et al.* Home monitoring of patients with cystic fibrosis to identify and treat acute pulmonary exacerbations. eICE Study Results. *Am J Respir Crit Care Med* 2017; 196: 1144–1151.
55 Sly PD, Brennan S, Gangell C, *et al.* Lung disease at diagnosis in infants with cystic fibrosis detected by newborn screening. *Am J Respir Crit Care Med* 2009; 180: 146–152.
56 Sly PD, Wainwright CE. Preserving lung function: the Holy Grail in managing cystic fibrosis. *Ann Am Thorac Soc* 2017; 14: 833–835.
57 Konstan MW, Morgan WJ, Butler SM, *et al.* Risk factors for rate of decline in forced expiratory volume in one second in children and adolescents with cystic fibrosis. *J Pediatr* 2007; 151: 134–139.
58 Sagel SD, Chmiel JF, Konstan MW. Sputum biomarkers of inflammation in cystic fibrosis lung disease. *Proc Am Thorac Soc* 2007; 4: 406–417.
59 Sagel SD, Kapsner RK, Osberg I. Induced sputum matrix metalloproteinase-9 correlates with lung function and airway inflammation in children with cystic fibrosis. *Pediatr Pulmonol* 2005; 39: 224–232.
60 Kim JS, Okamoto K, Rubin BK. Pulmonary function is negatively correlated with sputum inflammatory markers and cough clearability in subjects with cystic fibrosis but not those with chronic bronchitis. *Chest* 2006; 129: 1148–1154.
61 Mayer-Hamblett N, Aitken ML, Accurso FJ, *et al.* Association between pulmonary function and sputum biomarkers in cystic fibrosis. *Am J Respir Crit Care Med* 2007; 175: 822–828.
62 Ishak A, Stick SM, Turkovic L, *et al.* BAL inflammatory markers can predict pulmonary exacerbations in children with cystic fibrosis. *Chest* 2020; 158: 2314–2322.
63 Wojewodka G, De Sanctis JB, Bernier J, *et al.* Candidate markers associated with the probability of future pulmonary exacerbations in cystic fibrosis patients. *PLoS One* 2014; 9, e88567.
64 Wolter JM, Rodwell RL, Bowler SD, *et al.* Cytokines and inflammatory mediators do not indicate acute infection in cystic fibrosis. *Clin Diagn Lab Immunol* 1999; 6: 260–265.

65 Stoltz DA, Meyerholz DK, Welsh MJ. Origins of cystic fibrosis lung disease. *N Engl J Med* 2015; 372: 1574–1575.
66 Chen JH, Stoltz DA, Karp PH, *et al.* Loss of anion transport without increased sodium absorption characterizes newborn porcine cystic fibrosis airway epithelia. *Cell* 2010; 143: 911–923.
67 Stoltz DA, Meyerholz, DK, Pezzulo AA, *et al.* Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med* 2010; 2: 29ra31.
68 Horsley AR, Davies JC, Gray RD, *et al.* Changes in physiological, functional and structural markers of cystic fibrosis lung disease with treatment of a pulmonary exacerbation. *Thorax* 2013; 68: 532–539.
69 Gray RD, Imrie M, Boyd AC, *et al.* Sputum and serum calprotectin are useful biomarkers during CF exacerbation. *J Cyst Fibros* 2010; 9: 193–198.
70 Ordoñez CL, Henig NR, Mayer-Hamblett N, *et al.* Inflammatory and microbiologic markers in induced sputum after intravenous antibiotics in cystic fibrosis. *Am J Respir Crit Care Med* 2003; 168: 1471–1475.
71 Osika E, Cavaillon JM, Chadelat K, *et al.* Distinct sputum cytokine profiles in cystic fibrosis and other chronic inflammatory airway disease. *Eur Respir J* 1999; 14: 339–346.
72 Paats MS, Bergen IM, Bakker, M, *et al.* Cytokines in nasal lavages and plasma and their correlation with clinical parameters in cystic fibrosis. *J Cyst Fibros* 2013; 12: 623–629.
73 Bingle CD, Craven CJ. PLUNC: a novel family of candidate host defence proteins expressed in the upper airways and nasopharynx. *Hum Mol Genet* 2002; 11: 937–943.
74 Chu HW, Thaikoottathil J, Rino JG, *et al.* Function and regulation of SPLUNC1 protein in *Mycoplasma* infection and allergic inflammation. *J Immunol* 2007; 179: 3995–4002.
75 Gakhar L, Bartlett JA, Penterman J, *et al.* PLUNC is a novel airway surfactant protein with anti-biofilm activity. *PLoS One* 2010; 5: e9098.
76 Gally F, Di YP, Smith SK, *et al.* SPLUNC1 promotes lung innate defense against *Mycoplasma pneumoniae* infection in mice. *Am J Pathol* 2011; 178: 2159–2167.
77 Garcia-Caballero A, Rasmussen JE, Gaillard E, *et al.* SPLUNC1 regulates airway surface liquid volume by protecting ENaC from proteolytic cleavage. *Proc Natl Acad Sci USA* 2009; 106: 11412–11417.
78 McGillivary G, Bakaletz LO. The multifunctional host defense peptide SPLUNC1 is critical for homeostasis of the mammalian upper airway. *PLoS One* 2010; 5: e13224.
79 Britto CJ, Niu N, Khanal, S, *et al.* BPIFA1 regulates lung neutrophil recruitment and interferon signaling during acute inflammation. *Am J Physiol Lung Cell Mol Physiol* 2019; 316: L321–L333.
80 Thaikoottathil JV, Martin RJ, Di PY, *et al.* SPLUNC1 deficiency enhances airway eosinophilic inflammation in mice. *Am J Respir Cell Mol Biol* 2012; 47: 253–260.
81 Wright PL, Yu J, Di YP, *et al.* Epithelial reticulon 4B (Nogo-B) is an endogenous regulator of Th2-driven lung inflammation. *J Exp Med* 2010; 207: 2595–2607.
82 Sheikh S, Rahman M, Gale, Z, *et al.* Differing mechanisms of leukocyte recruitment and sensitivity to conditioning by shear stress for endothelial cells treated with tumour necrosis factor-α or interleukin-1β. *Br J Pharmacol* 2005; 145: 1052–1061.
83 Ning F, Wang C, Berry KZ, *et al.* Structural characterization of the pulmonary innate immune protein SPLUNC1 and identification of lipid ligands. *FASEB J* 2014; 28: 5349–5360.
84 Wagener JS, Rasouliyan L, VanDevanter, DR, *et al.* Oral, inhaled, and intravenous antibiotic choice for treating pulmonary exacerbations in cystic fibrosis. *Pediatr Pulmonol* 2013; 48: 666–673.